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Title: Early Development of the Endocrine System in Salmonids: Regulation of Sex Differentiation and Ontogeny of the Stress Response.

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Carl B. Schreck

Sex steroids, follicle stimulating hormone (FSH), luteinizing hormone (LH) and gonadotropin releasing hormone (GnRH) were measured in monosex and mixed sex populations of rainbow trout during early development. Steroids declined following fertilization, increased at hatch, then fell and remained constant thereafter. Trends toward differences in steroids between males and females became evident around the time of gonadal differentiation. FSH and GnRH were always detectable whereas LH was not. Peptide hormones were not sexually dimorphic. Given that the brain-pituitary-gonadal axis appears to be intact during the process of sexual differentiation and the dynamics of steroid levels during this process, sex steroids may drive sexual differentiation of rainbow trout.

Gynogenetic rainbow trout were sex-reversed to produce XX males by using 17α-methyltestosterone (MT) and 11β-hydroxyandrostenedione (OHA). Steroids were administered by

immersion or immersion plus feeding to determine if males with intact sperm ducts could be produced. Immersion in MT resulted in varying degrees of masculinization while immersion plus feeding produced nearly 100% males. The most effective period for steroid immersion was one week post-hatch. Immersion in OHA caused low rates of masculinization, while immersion plus feeding resulted in 70% males. Males produced through both immersion and feeding of MT generally did not develop sperm ducts; whereas animals treated by immersion alone in MT, or those produced with OHA, tended to be functional. Cryopreserved semen from functional males produced 100% female populations.

Cortisol was measured in chinook salmon during early development in both stressed and non-stressed fish to determine when the corticosteroidogenic stress response first arises. Levels of cortisol were low in eyed eggs, increased at hatch, decreased 2 weeks later and then remained constant thereafter. Differences in cortisol between stress and control fish were found 1 week after hatch and persisted for the remainder of the study. A decrease in the ability to elicit cortisol was seen 4 weeks after hatch. The decreases in both endogenous cortisol content and magnitude of the stress response may be comparable to developmental events of mammals where corticosteroid synthesis is inhibited to neutralize possible detrimental effects of these hormones during critical periods of development.

Early Development of the Endocrine System in Salmonids: Regulation of Sex Differentiation and Ontogeny of the Stress Response.

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Grant William Feist

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

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Grant William Feist, Author

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CONTRIBUTION OF AUTHORS

Choo-Guan Yeoh and Dr. Martin Fitzpatrick appear as coauthors of chapter III. I thank them for their assistance in experimental design and interpretation of results.

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I: INTRODUCTION

BACKGROUND

Sex differentiation is the process by which genetic sex is translated into phenotypic sex. Although far from complete, many of the molecular and endocrine mechanisms of mammalian sex differentiation have been elucidated. In the absence of a Y chromosome, organisms follow a preprogrammed path of female differentiation where the primordial gonad becomes an ovary which subsequently directs the formation of secondary sex characteristics (Nagai, 1992). In the presence of the Y chromosome, gene products are expressed which dictate the formation of a testis which then secretes hormones that inhibit female development and bring about male secondary characteristics (Jost et al., 1973).

The testis determining factor, long believed to be present on the Y chromosome, was finally discovered by Sinclair *et al.* (1990) and was termed the SRY gene (sex-determining region Y). SRY is responsible for initiating the cascade of events which results in regression of the female (Mullerian) ducts of the gonad with the ultimate formation of Wolffian (male) ducts resulting in a testis. SRY does this by an unknown mechanism in which undifferentiated cells become sertoli cells which secrete anti-Mullerian hormone

that inhibits female duct formation. Further differentiation of cells in the gonad results in the formation of Leydig cells which then secrete testosterone which masculinizes the Wolffian duct (Wachtel and Tiersch 1994). The final result is a functional testis which will secrete the appropriate androgens necessary for development of secondary sex characteristics including masculinization of the central nervous system (MacLusky and Naftolin, 1981; McEwen, 1981).

Unlike mammals, very little is known about either genetic or endocrine mechanisms for control of sex differentiation in fish. Compared to other vertebrates, fish display the widest array of both sex determining mechanisms and reproductive strategies. These strategies include almost all ranges of various types of hermaphroditism to gonochorism (Yamamoto, 1969). Genetic sex determining mechanisms include XX/YY and ZZ/ZW systems as well as autosomal, polygenic and multiple sex chromosomes. Other influences include both social and environmental factors (see reviews by Nakamura *et al.*, 1998; Baroiller *et al.*, 1999). Although some researchers have identified SRY-like sequences in fish, no functional testis determining factor has been found (Fukada *et al.*, 1995; Takamatsu *et al.*, 1997; Baroiller *et al.*, 1999). Given the incredible plasticity with regard to reproduction in fish it is not surprising that it is extremely difficult to study possible mechanisms of sex differentiation.

Exposure of organisms to exogenous sex steroids during early developmental stages can result in varying degrees of sex reversal in a variety of fishes (see reviews by Yamamoto, 1969; Schreck, 1974; Donaldson and Hunter, 1982; Hunter and Donaldson, 1983). Application of androgens usually results in masculinization while exposure to estrogens results in feminization. This ability of steroids to cause sex inversion in the medaka, *Oryzias latipes*, led Yamamoto (1962, 1969) to propose his theory that sex steroids are the natural inducers of sexual differentiation in fishes.

Several studies have been conducted which examined steroidogenic capabilities of differentiating teleostean gonads. Work with radioactive steroid precursors has shown the presence of steroid synthesizing enzymes during early development for the guppy, *Poecilia reticulata*, (Takahashi and Iwasaki, 1973) and the rainbow trout, *Oncorhynchus mykiss*, (van den Hurk *et al.*, 1982; Antila, 1984; Yeoh *et al.*, 1996a). Using *in vitro* techniques, Fitzpatrick *et al.* (1993) showed that there were differences between male and female rainbow trout for the ability to produce androgens and estrogen after gonadotropin stimulation but that this difference appeared around the time that gonads showed obvious signs of differentiation. They also found that interrenal tissue was capable of synthesizing steroids earlier than gonads and suggested that extra-gonadal influence on differentiation should not be discounted.

Rowell *et al.* (1999) used *in vitro* techniques with tilapia, *Oreochromis niloticus*, and found that males produced 2 to 3 times more testosterone (T) than females during gonadal differentiation.

Studies have examined endogenous sex steroid content during early development of chum salmon, *O. keta* (de Jesus and Hirano, 1992) and rainbow trout (Yeoh et al., 1996b) but they did not address the process of sex differentiation. Yeoh *et al.* (1996a,b) found that trout alevins could produce glucuronide conjugates from both endogenous and exogenous steroids and suggested that this type of steroid metabolism may be important for early embryonic development.

Rothbard *et al.* (1987) measured endogenous steroid levels during the process of sexual differentiation in three species of tilapia. They found that T and 17β -estradiol (E2) were present in ng/individual concentrations just after fertilization and declined to undetectable levels in fry 3-4 weeks old. The concentrations of both steroids began to increase in fry 4-6 weeks old. The increase of T was 10-fold higher than that of E2. They concluded that, among the steroids examined, only T reflected the process of sexual differentiation.

Work in our laboratory with coho salmon, *O. kisutch*, has shown whole body increases of T, 11-ketotestosterone, androstenedione and E2 around the time of hatching which was accompanied by possible bimodal distributions of these steroids (Feist *et al.*, 1990). Recently, all-female and all-

male populations of tilapia were immunohistochemically examined using antibodies specific to steroid synthesizing enzymes during early development (Nagahama, 1999). The researchers were able to detect the enzymes in all-female fry 10-15 days before morphological sex differentiation was evident. Immunoreactive cells then increased in number as ovarian development progressed. Enzymes were not detected in all-male fry either before or during testis formation but did appear later. Profiles of immunoreactive aromatase suggested that estrogens are responsible for female differentiation in tilapia. Using these techniques in rainbow trout, Guiguen *et al.* (1999) found results for both males and females to be similar, demonstrating possible species differences with regard to testis differentiation. These findings suggest that sex steroids may play a role in the process of sexual differentiation in some fish before this event is histologically discernible.

In addition, work with aromatase inhibitors has shown that female sex differentiation is dependent on estrogen synthesis in chinook salmon, *O. tshawytscha* (Piferrer *et al.*, 1994), rainbow trout (Guiguen *et al.*, 1999) and tilapia (Nakamura et al., 1999). These studies provide further evidence that steroids are playing some role during the process of sex differentiation.

Sex steroid receptors have been found in the brains from a variety of fishes (including salmonids) in regions similar to those found in mammals (Kim *et al.*, 1978; Morrell

and Pfaff, 1978) and aromatase has also been detected in the brain and pituitary of several teleosts (Pasmanik and Callard, 1985; Timmers *et al.*, 1987). Androgen receptors have been reported in ovaries of juvenile coho salmon (Fitzpatrick *et al.*, 1994) and in mature testis and immature tilapia ovaries (Gale *et al.*, 1996). The presence of gonadal receptors may not only provide possible explanations for mechanisms of exogenous steroid sex reversal, but may offer other potential models of sex differentiation.

The central nervous system of fishes appears to be similar to other vertebrates with regard to distribution of protein hormones. Pituitary gonadotrophs have been documented for several species (Olivereau and Nagahama, 1983; Dubourg *et al.*, 1985) including neonates (van den Hurk, 1982; Schreibman *et al.*, 1982). Gonadotropin-releasing hormone (GnRH) has been located in the brain and pituitary of fishes in areas comparable to that of mammals (Crim *et al.*, 1979; Schreibman *et al.*, 1979, 1982; Munz *et al.*, 1981; Kah *et al.*, 1984).

Two chemically distinct forms of gonadotropin (originally termed GTH I and II but now recognized as follicle stimulating hormone-FSH, and luteinizing hormone-LH) have been identified in chum salmon (Suzuki *et al.*, 1988a), coho salmon (Swanson *et al.*, 1987), rainbow trout and Atlantic salmon, *Salmo salar*, (Nozaki *et al.*, 1990a). Specific antibodies to these two hormones have been produced which offer a variety of research possibilities concerning

both pituitary control of gonadal steroid synthesis and the process of sexual differentiation. Only FSH is present in sexually immature fish and both FSH and LH are seen in mature animals (Nozaki *et al*, 1990b). LH also appears to be more potent than FSH in stimulating maturational hormone synthesis (Suzuki *et al.*, 1988b).

Several studies have examined early development of the brain-pituitary-gonadal axis (BPGA) in fishes but they did not address the process of sexual differentiation. Immunocytochemical identification of GnRH has been shown for larval western brook lampreys, *Lampetra richardsoni*, (Crim *et al.*, 1979) and red seabream, *Pagras major*, (Ookura *et al.*, 1999). van den Hurk (1982) found that GTH was present in rainbow trout during early development and showed that the addition of 17α-methyltestosterone (MT), progesterone and 17α-hydroxyprogesterone stimulated the development of GTH cells shortly after hatching while treatment with 11β-hydroxyandrostenedione did not.

Work with neonatal platyfish, *Xiphophorus maculatus*, has shown the presence of both GTH and GnRH (Schreibman *et al.*, 1982). The ontogeny of GnRH appearance in the brains of platyfish has also been examined (Halpern-Sebold and Schreibman, 1983). These researchers found differences in the timing of appearance and localization of this hormone in genetically determined late and early maturing males. GnRH was first produced in the nucleus olfactoretinalis and nucleus preopticus periventricularis (NPP) of sexually

immature fish. At the time of puberty the hormone was found in the NPP and also in the nucleus lateralis tuberis pars posterior. Development of the areas of the brain containing GnRH was sequential and occurred before the appearance of pituitary gonadotrophs and maturation of the gonads. They therefore concluded that the appearance of GnRH was essential for further development of the BPGA and subsequent maturation. Schreibman *et al.* (1985) have also shown a sexual dimorphism in the distribution and intensity of staining for GnRH in sexually mature animals for this species.

The ontogeny of the pituitary gland has been studied in coho salmon (Mal *et al.*, 1989), sea bass, *Dicentrarchus labrax* (Cambre *et al.*, 1990), and chum salmon (Naito *et al.*, 1993). In chum salmon five pituitary hormones; prolactin (PRL), thyroid stimulating hormone (TSH), melanocyte stimulating hormone (MSH), adrenocorticotrophic hormone (ACTH) and growth hormone (GH) cells were identified between 28 and 42 days postfertilization (dpf). FSH cells were first detected at 56 dpf and LH cells appeared around the time of puberty. In the sea bass, cells immunoreactive for TSH, GH, and ACTH were visible the day after hatching , PRL was observed between 9 and 15 days posthatch and mammalian LH was not seen during the first 26 days after hatch. For the chum salmon the five hormones seen in the coho study first appeared in eyed eggs five weeks after fertilization, but FSH and LH were not detected even 3 weeks after hatching. It

was also found that neuroendocrine fibers from both the hypothalamus and preoptic areas began to grow and reached the neurohypophysis one week before hatch. These and the previous studies indicate that both hypothalamic and pituitary hormones are present very early in development and that species differences most likely exist.

Studies concerned with endocrine influence on sexual determination in the lower vertebrates can perhaps lead to a better understanding of how this process and its control arose evolutionarily in the higher vertebrates. There may be potential for development of a model system for understanding human sexual differentiation which may prove valuable in determining causes of infertility and other sexual disorders.

Information obtained from the study of endocrine control of sexual differentiation can also be applied to developing efficient methodologies for the production of monosex or sterile populations of salmonids which are potentially useful for several reasons. All female populations could increase egg availability for both aquaculture systems and the export market. All male populations of some aquaculturally important species (e.g. tilapia) have higher growth rates than bisexual groups. Sterile salmon do not mature sexually, thus the common problem of precocious development of male salmon could be eliminated. Sterile salmon continue to grow during, and sometimes for several years after, the period of normal

maturation and also retain high quality flesh characteristics unlike maturing fish. Sterile populations also remain available to ocean fisheries beyond the time when normal fish return to natal streams to spawn and die (Donaldson and Hunter, 1982; Hunter and Donaldson, 1983). Thus, the economic value of sterile fish may exceed that of normal fish. Finally, sterile salmon may be released into natural environments without risking genetic contamination of natural populations. We caution against this practice, however, because techniques to produce monosex or sterile populations are rarely 100% effective.

It has become common practice in aquaculture systems to use exogenous steroid administration to reverse the sex of a great variety of fishes (Pandian and Sheela, 1995). The most widely used technique is the feeding of steroids to fish, usually for long periods (30-60 days), and involves relatively high doses (mg steroid/kg feed). The steroid typically used for masculinization is MT but this technique results in varying degrees of sex reversal and may expose workers to high levels of potentially harmful compounds (Green *et al.*, 1997). Feeding of MT allows for the production of masculinized female salmonids (XX males) as a source of broodstock to sire all-female populations (Bye and Lincoln, 1981; Hunter *et al.*, 1982; Hunter and Donaldson, 1983). This procedure can produce high percentages of males but the majority of the fish lack sperm ducts and sperm must be removed surgically (Bye and Lincoln, 1981). Both feeding

of steroids and surgical procedures are time consuming and may be detrimental to broodstock.

A possible alternative to feeding is immersing fry in steroids for brief periods during early development. This technique has been used to produce both sex-reversed male and female salmonids (Goetz *et al.*, 1979; Hunter *et al.*, 1982, 1986; van den Hurk and van Oordt, 1985; Piferrer and Donaldson, 1989, 1991, 1992, 1993). Steroids are applied around the time of hatching for a period as short as two hours. There appear to be discrete developmental windows which occur approximately one week apart where fish are susceptible to either masculinizing or feminizing effects of steroids (Piferrer and Donaldson, 1989). The use of immersion techniques for sex reversal are both time and cost effective and may be less detrimental to both fish and workers in aquaculture facilities. Lower concentrations of steroids applied for brief periods may result in higher proportions of animals with intact sperm ducts, thus obviating the need for surgical removal of sperm, and may also produce lower proportions of sterile fish.

Similar to the situation for sex determining mechanisms, the ontogeny of the corticosteroidogenic stress response has been well documented in mammals (see reviews by Sapolsky and Meaney, 1986; Carsia and Malamed, 1989) but few studies have been conducted in fish. In mammals the hypothalamic-pituitary-adrenal axis is activated during late fetal stages of development. Shortly after birth the

corticosteroidogenic stress response becomes markedly reduced and organisms are unable to elicit corticosteroids following a stressful event. This developmental time has been termed the stress hyporesponsive period (SHRP) and is thought to be an adaptive mechanism to decrease the deleterious effects that corticosteroids can have on central nervous system development (Schapiro, 1962; Sapolsky and Meaney, 1986; de Kloet *et al.*, 1988).

Cortisol is the main corticosteroid produced by the majority of fishes in response to a stressor. This response is believed to be of adaptive value in the short term but prolonged stress and/or elevated cortisol levels can have deleterious effects (Barton and Iwama, 1991). Detrimental effects of cortisol have been documented for survival (Specker and Schreck, 1980), growth (McCormick *et al.*, 1998), reproduction (Campbell *et al*, 1994), immune function (Maule *et al.*, 1989) and general fitness (Schreck, 1982). Information related to when fish develop the ability to respond to stress and at which times during development that they are particularly sensitive to stressors could lead to handling methodologies which are more conducive to fitness in both aquaculture and wild systems.

Many studies have examined cortisol content of developing fish embryos in a variety of species (de Jesus *et al.*, 1991; de Jesus and Hirano, 1992; Hwang, P.P. *et al.*, 1992; Barry *et al.*, 1995a; Sampath-Kumar *et al.*, 1995,1997; Yeoh *et al.*, 1996), but only three have examined when

organisms can first elicit cortisol in response to a stress. Barry *et al.* (1995a,b) were able to detect a stress response in rainbow trout at 42 dpf or two weeks after hatch while Pottinger and Mosuwe (1994) documented a corticosteroidogenic response in rainbow and brown trout, *S. trutta*, 5 weeks after hatching.

Barry et al. (1995a,b) also found that the ability of young rainbow trout to produce cortisol following a stress decreased approximately four weeks after hatching and compared this to the SHRP seen in mammals. They also suggested that subtle manipulations of fish during this time could possibly lead to stress-resistant strains. There is evidence that the variation in the magnitude of the stress response, or ability to synthesize cortisol, is genetically based (Pottinger *et al.*, 1994) and that it may be possible to use selective breeding protocols to decrease the stress responsiveness of fish for intensive aquaculture purposes (Fevolden *et al.*, 1991; Pottinger and Pickering, 1997; Pottinger and Carrick, 1999).

GOALS AND OBJECTIVES

The goal of this work is to examine the development of the endocrine system in salmonids as a means of elucidating possible endocrine control mechanisms of both sexual differentiation and the corticosteroidogenic stress response.

The objectives of this research were:

1) To determine if all-female, all-male, and bisexual populations of rainbow trout differ with regard to the amounts of androgens and estrogen synthesized during sexual differentiation and development.
2) To determine if all-female, all-male and bisexual populations differ with regard to the amount or localization of FSH, LH and GnRH in the brain and pituitary during sexual differentiation and development.
3) To determine if exposure to exogenous steroids during early development can both sex reverse rainbow trout and produce functional males.
4) To determine when during development the corticosteroidogenic stress response first appears in chinook salmon.
5) To determine if progeny from either pooled or individual females differ with regard to the timing or magnitude of the stress response.

ORAGNIZATION OF THE THESIS

This thesis is organized into five chapters. Chapter II examines BPGA development in rainbow trout. Findings suggest that sex steroids may play the driving role during sex

differentiation. Chapter III looks at the effects of exogenous steroid administration on sexual differentiation and maturation of rainbow trout. Findings suggest that functional "XX" males can be produced when the appropriate steroid is exposed to fish during specific developmental windows. Chapter IV examines when in development the stress response develops. The results of this study showed that a stress response is first detectable one week after hatching and that patterns between progeny from both pooled and individual females showed remarkable similarity. Chapters II through IV are all presented in manuscript form. The fifth chapter is a general summary with conclusions.

II. BRAIN-PITUITARY-GONADAL AXIS DURING EARLY DEVELOPMENT AND SEXUAL DIFFERENTIATION IN THE RAINBOW TROUT, *Oncorhynchus mykiss*[1]

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ABSTRACT

Profiles of testosterone, 11-ketotestosterone, androstenedione, and estradiol were determined by RIA, and immunocytochemical techniques were employed to identify FSH, LH and gonadotropin releasing hormone (GnRH) in monosex and mixed sex populations of rainbow trout from 1 to 126 days postfertilization (dpf). Steroid levels were relatively high at 1 dpf and declined until 25 dpf. At 30 and 48 dpf (hatching) steroid levels increased slightly before they fell by 78 dpf and remained relatively constant thereafter. Trends toward differences in steroid content between males and females became evident around the time gonadal differentiation was histologically discernible (78 and 90 dpf). FSH was present in the proximal pars distalis at all dates (48-126 dpf) whereas LH was not detectable. GnRH was found at all dates (48-126 dpf) and was distributed in several areas of the brain including the nucleus preopticus periventricularis, nucleus lateralis tuberis, and the pituitary in the region where FSH was found. No differences were seen between males and females in the timing of appearance, localization, or intensity of staining of these peptide hormones. Given that the brain-pituitary-gonadal axis seems to be intact during the process of sexual differentiation and the dynamics of steroid levels during this process, sex steroids may play the driving role for sexual differentiation of rainbow trout.

INTRODUCTION

The attainment of sexual maturation and fertility can be viewed as a continuum from sexual differentiation of the fetus and the development of the brain-pituitary-gonadal axis (BPGA) to maturation at puberty (Grumbach *et al.*, 1974). The administration of sex steroids during early development results in varying degrees of sex reversal in a variety of teleostean fishes (see reviews by Yamamoto, 1969; Schreck, 1974; Donaldson and Hunter, 1982; Hunter and Donaldson, 1983). Androgens typically act as andro-inducers and estrogens, as gyno-inducers. By virtue of this ability to cause sex inversion in the medaka, *Oryzias latipes*, Yamamoto (1962, 1969) advanced the theory that sex steroids are the natural inducers of sexual differentiation in fishes. There is insufficient evidence to determine whether these steroidal effects involve a permanent developmental change in the central nervous system (CNS) or if it is merely hormone-induced differentiation of peripheral structures (MacLusky and Naftolin, 1981)

Few studies have been done to examine the onset of steroidogenesis in the differentiating teleostean gonad. The administration of radioactive steroid precursors demonstrated the presence of steroid synthesizing enzymes during the early life stages of the guppy, *Poecilia reticulata*, (Takahashi and Iwasaki, 1973) and the rainbow trout, *Oncorhynchus mykiss* (van den Hurk *et al.*, 1982;

Antila, 1984; Yeoh *et al.* 1996a). Work in our laboratory also demonstrated the ability of rainbow trout embryos to synthesize steroid glucuronides from both maternal and exogenous origins (Yeoh *et al.*, 1996 a,b) and the ability of gonads and interrenals to respond to gonadotropin (Fitzpatrick *et al.*, 1993). Rothbard *et al.* (1987) examined endogenous steroid levels during sexual differentiation in three species of tilapia and concluded that among the examined steroids only testosterone reflected the process of sexual differentiation.

We completed work with coho salmon, *O. kisutch*, which showed increases in whole body content of testosterone (T), 11-ketotestosterone (KT), 17β-estradiol (E2), and androstenedione (A) shortly after hatching; concentrations of these steroids seemed to be bimodal in populations sampled around hatching (Feist *et al.*, 1990). Yeoh *et al.* (1996b) showed increases in E2 and KT content of steelhead trout around the time of hatch. They also found differences in KT between gynogenetic and control (mixed sex) groups throughout most of early development. These findings suggest that dimorphisms in hormone content may appear during very early development and that sex steroids may have a role in the process of sexual differentiation in salmonids before this event is histologically discernible.

Protein hormone content in the CNS of fishes seems to be similar to that found in other vertebrate groups. Gonadotropin cells have been located in the pituitaries of

several species (Olivereau and Nagahama, 1983; Dubourg *et al.*, 1985) including neonates (van den Hurk, 1982; Schreibman *et al.*, 1982). Gonadotropin-releasing hormone (GnRH) has also been found in the brain and pituitary of fishes in regions similar to those of mammals (Crim *et al.*, 1979; Schreibman *et al.*, 1979, 1982; Munz *et al.*, 1981; Kah *et al.*, 1984).

Two chemically distinct forms of gonadotropin (originally termed GTH I and II but now recognized as follicle stimulating hormone-FSH and luteinizing hormone-LH) have been documented in salmonid pituitaries for chum salmon, *O. keta*, (Suzuki *et al.*, 1988a), coho salmon (Swanson *et al.*, 1987), rainbow trout, and Atlantic salmon, (Nozaki *et al.*, 1990a). Specific antibodies to these two forms of are available and offer research into the pituitary control of gonadal steroid synthesis in both teleosts and higher vertebrate groups. In sexually immature fish only FSH is present whereas in mature animals LH is also present (Nozaki *et al*, 1990b) and is more potent than FSH in stimulating maturational hormone (DHP) synthesis (Suzuki *et al.*, 1988b). Other than a study by Mal *et al.*, (1989) on coho salmon, no researcher has examined whether LH is present during early developmental stages.

Several researchers examined early development of the BPGA in fishes, yet none addressed the process of sexual differentiation. Faint immunocytochemical staining of GnRH has been observed in larval Western Brook Lampreys, *Lampetra*

richardsoni, (Crim *et al.*, 1979). Van den Hurk (1982) found immunoreactive GtH in rainbow trout at 50 days postfertilization (dpf) and also observed that addition of methyltestosterone, progesterone, and 17α-hydroxyprogesterone
stimulated the development of GtH cells shortly after hatching whereas 11β-hydroxyandrostenedione did not. Both GtH and GnRH have been localized in neonatal platyfish, Xiphophorus maculatus, (Schreibman *et al.*, 1982). Halpern-Sebold and Schreibman (1983) traced the ontogeny of GnRH in the brains of platyfish and found differences in the timing of appearance and localization of this hormone in genetically determined late and early maturing males. They concluded that the appearance of GnRH in the brain is essential for further development of the BPGA and subsequent maturation. A sexual dimorphism in the distribution and intensity of staining of GnRH in sexually mature males and females of this species has also been demonstrated (Schreibman *et al.*, 1985).

The ontogeny of the pituitary gland in coho salmon was studied by Mal *et al.* (1989). At 28 dpf, prolactin (PRL) cells were identified. Between 35 and 42 dpf, thyroid stimulating hormone (TSH), melanocyte stimulating hormone, adrenocorticotrophic hormone (ACTH), and growth hormone (GH) cells were Identified. FSH cells were present at 56 dpf, and LH cells did not appear until near the time of puberty. Work with the seabass, Dicentrarchus labrax, revealed that cells

immunoreactive for TSH, GH, and ACTH were visible the day after hatching, and PRL was observed between 9 and 15 days posthatch (Cambre *et al.*, 1990). Immunoreactive mammalian luteinizing hormone was not seen during the first 26 days after hatch.

Our objective was to elucidate possible endocrine control mechanisms of sexual differentiation in salmonids by evaluating development of the BPGA and sex steroid profiles in control (normal mixed sex) and monosex populations of rainbow trout through the period of sexual differentiation and by correlating hormonal events with gonadal development. The use of monosex groups of fish is advantageous in studies of this nature because the sex of individuals is known before gonadal differentiation occurs. Steroid profiles of whole body extracts were generated by radioimmunoassay (RIA) specific for T, KT, A, and E2. Immunocytochemical techniques were employed to identify FSH and LH in the pituitary and GnRH in the brain to determine BPGA development and protein hormone synthesizing capabilities during early development and sexual differentiation.

MATERIALS AND METHODS

We examined populations of rainbow trout from 2 brood years. Fish from the first brood year in (hereafter referred to as experiment 1) were analyzed for whole body sex steroid content and gonadal development to ascertain the period of

sexual differentiation and determine dynamics in steroid levels. Fish from the second brood year (hereafter referred to as experiment 2) were also analyzed for protein hormone distribution in the brain and pituitary.

Fish Rearing and Sampling

Monosex and control populations of rainbow trout for experiment 1 were obtained from Dr. G. Thorgaard's laboratory at Washington State University, Pullman, Washington. All female populations were created by gynogenesis (Thorgaard *et al.*, 1983) and all male groups through the use of YY sperm. The eggs were incubated at Oregon State University's (OSU) Fish Genetics and Performance Laboratory at Smith Farm, Corvallis, at a constant water temperature of 12 °C. Gametes for experiment 2 were obtained from OSU's department of food science and technology. YY sperm was obtained from Dr. Thorgaards laboratory. Eggs from 12 females and sperm from 3 males were transported on ice to OSU's Nash Hall where gynogenesis was performed. Pooled rainbow trout sperm (1 ml) was diluted 1:10 in 0.3 M glucose, poured into 16 x 25.5 cm glass trays and irradiated at 400 μw/cm2 for 2.5 min with UV light. Pooled eggs (approximately 3000) were poured directly into the trays with the inactivated sperm, mixed with water, and allowed to sit for 2 min before being rinsed and allowed to sit for another 8 min. Eggs were then heat shocked at 29 °C for 10 min in a circulating water bath. Normal, mixed sex

controls were produced in the same manner except that sperm was not irradiated. YY sperm to produce an all male population was again obtained from Washington State University. The eggs were incubated at Nash Hall at a water temperature of 8.5 °C and were then transported as eyed eggs to Smith Farm. Biomoist diet was fed from the onset of exogenous feeding 3 or 4 times daily to satiation.

Rainbow trout eggs from experiment 1 were sampled at 1 hr postfertilization and at 5, 10, 15, 20, 32, 39, 46, 53, 60, and 67 days postfertilization (dpf). Fish from experiment 2 were sampled at 1 hr postfertilization and at 10, 15, 20, 25, 30, 48, 78, 90, 100, 111, and 126 dpf. The number of remaining fish from experiment 2 after hatching (48 dpf) was limited; sampling was therefore discontinued until near the time of presupposed gonadal differentiation (78 dpf) to conserve fish. A sample size of 40 embryos was taken on each date. We determined steroid levels in 30 fish and fixed 10 fish in Bouin's solution for histological analysis. An additional 10 fish were sampled at each date in experiment 2 after hatching (48 dpf) for immunocytochemical analyses. All reported values of steroid levels are for sample sizes of 30.

Extractions

Embryos to be analyzed for whole-body sex steroid content were extracted with a method by Feist *et al.* (1990). The average extraction efficiencies of steroids ranged from

51.8 to 67.1%. All steroid assays were corrected for recovery.

Assays

Whole body sex steroid content for T, KT, and E2 were measured by RIA as described by Sower and Schreck (1982) and modified by Fitzpatrick *et al.* (1986) and for A as described by Feist *et al.* (1990). A slightly more concentrated charcoal solution (6.25 g charcoal and 4.0 g dextran/liter PBSG) was used for all assays to reduce non-specific binding. We added 1.0 ml of dextran coated charcoal to each tube for the T, KT, and A assays and 0.5 ml for the E2 assay. The lower limit of detection was 3.1 pg/tube for all assays except E2, which was 1.25 pg/tube. The intra-assay coefficient of variation for all assays was less than 5%. Steroid levels determined by RIA were validated by verifying that serial dilutions were parallel to standard curves. Authentic steroids have also been verified in eggs and embryos of coho salmon by high performance liquid chromatography and thin layer chromatography (Feist *et al.*, 1990).

Immunocytochemistry

Whole fish were fixed in Bouin-Hollande sublimate for 12 hr. Fish heads were then dehydrated through a series of increasing concentrations of ethanol. After 90% ethanol, the

fish were washed in a solution of potassium iodide in 90% ethanol for 24 hr to remove deposits of mercuric chloride. Samples were embedded in paraplast, and serial sagittal sections (4-10 μm) were mounted on glass slides. The rabbit anti-coho salmon FSH and LH antibodies were raised by P. Swanson (Swanson *et al.*, 1987). The salmon GnRH antibodies were obtained from N. Sherwood at the University of Victoria, British Columbia.

Immunocytochemical staining was performed with a Vectastain ABC (Avidin-biotin-peroxidase complex) kit following a procedure by Nozaki *et al.*, (1988). After rehydration, deparaffinized glass slide mounted sections were incubated for 15 min in 0.3% hydrogen peroxide in methanol to block endogenous peroxidase activity. Sections were incubated with 5% normal goat serum for 30 min to reduce nonspecific staining and then washed in phosphate buffered saline (PBS: 0.14 M NaCL, 0.01 M phosphate buffer, pH 7.4). Primary antibodies diluted at 1:1000 were applied to the sections for 24 hr at 4 °C, then rinsed in PBS for 20 min. Vectastain goat biotinylated anti-rabbit immunoglobulin and Vectastain ABC reagent were each applied for 1 hr and followed by 10 min rinses in PBS. Finally, 3,3'-diaminobenzidine tetrahydrochloride and 0.003% hydrogen peroxide were used for visualization of the immunoreaction. All incubations, except for the primary antibodies, were at room temperature in closed humid containers.

Control procedures for testing the specificity of the reactions were done by 1) incubating sections with only antibody diluant, 2) replacing the primary antibodies with normal rabbit serum, or 3) using primary antibodies that had been absorbed by incubating them for 24 hr at 4 °C with 2 µg/ml of purified antigen. The nomenclature for areas of the brain containing GnRH is from Billard and Peter (1982).

Histology

Fish sampled for histological analysis were fixed in Bouin's solution and embedded in paraffin. Serial sections (4 µm) were taken sagittally and stained with hematoxylin and eosin. Descriptions of gonadal differentiation of salmonids outlined by Goetz et al. (1979), Robertson (1953), Ashby (1957), Takashima *et al.* (1980), and van den Hurk and Slof (1981) were used to identify stages of germ cell and gonadal differentiation.

Statistics

Data for sex steroid content between all-female, all-male, and control populations were analyzed by the nonparametric Mann-Whitney rank sum statistic. The level of significance for all tests was $P < 0.05$.

Because bimodal distributions of sex steroid concentrations in developing embryos are a possible indication of sexual dimorphism, data for sex steroid distributions for each sampling date were also analyzed for

skewness (g1) and kurtosis (g2) following Sokal and Rohlf (1969). The ratio of g1 or g2 to their standard errors is a test of normality. Formulas for standard errors of g1 and g2 are from Cramer (1946).

RESULTS

A conceptual framework of developmental events that includes hormone dynamics for experiments 1 and 2 is given in Fig. 1.

Steroid content of postfertilized eggs

Concentrations of all examined steroids during both experiments were relatively high in 1 hr old postfertilized eggs from the control group (Fig. 2). The pattern of sex steroid content was also similar between brood years. Relatively large amounts of T and A and smaller amounts of KT and E2 were present.

Post-fertilized eggs to 20 dpf, Experiment 1.

Steroid levels were relatively high in eggs at 1 hr post-fertilization, but declined precipitously in all three groups of fish by 20 dpf (Fig. 3). Steroids in male and female groups did not tend to differ much during this period except levels of E2 were higher in males than females at 5 dpf and levels of T were lower in males than females at 10 dpf. Male and female groups differed from controls at several sampling dates. Females had lower levels of E2 at 5

Figure 1. Conceptual representation of sampling times, developmental events, steroid levels, gonadal development, and presence of peptide hormones in populations of control, female, and male rainbow trout from two brood years. Steroid levels conceptually depicted by the height of the bars are not drawn to scale.

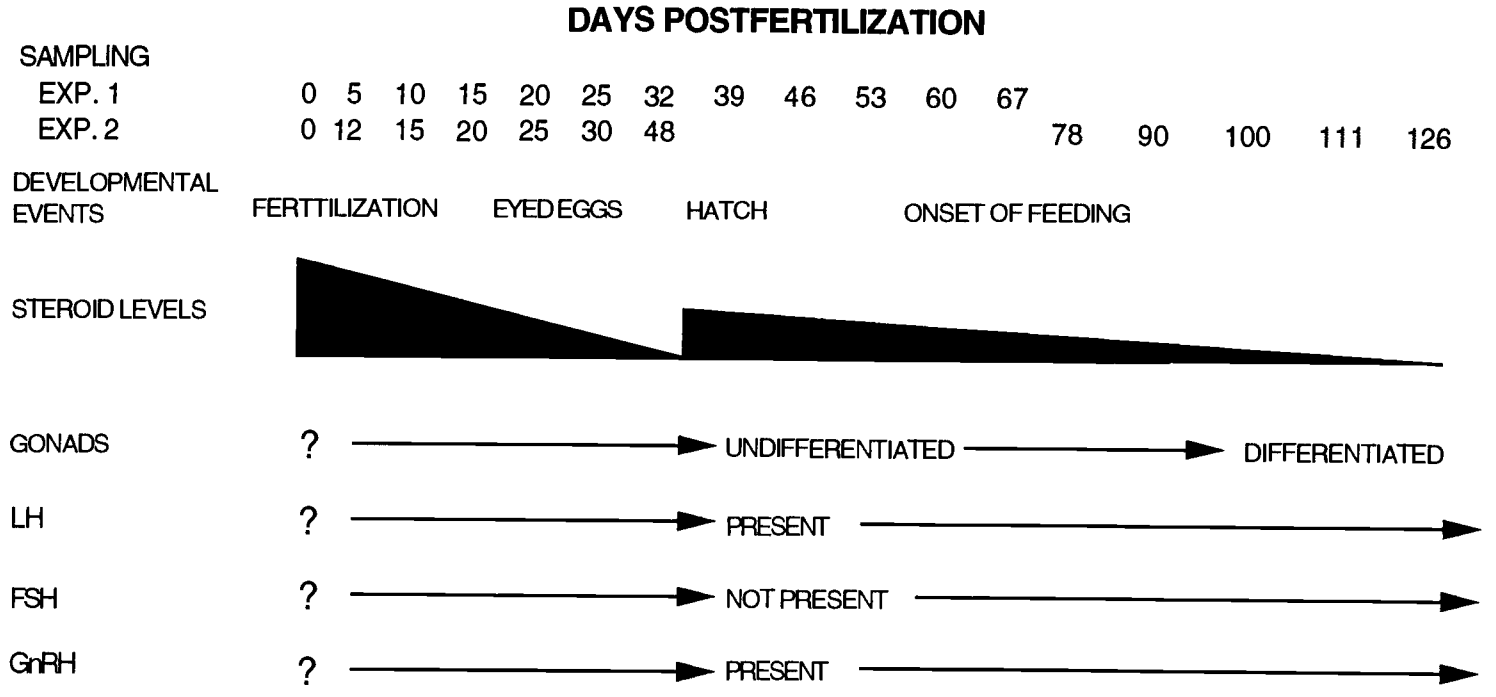
DAYS POSTFERTILIZATION
SAMPLING
EXP. 1 0 5 10 15 20 25 32 39 46 53 60 67
EXP. 2 0 12 15 20 25 30 48 78 90 100 111 126
DEVELOPMENTAL EVENTS
FERTTILIZATION
EYED EGGS
HATCH
ONSET OF FEEDING
STEROID LEVELS
GONADS
?
UNDIFFERENTIATED
DIFFERENTIATED
LH
?
PRESENT
FSH
?
NOT PRESENT
GnRH
?
PRESENT

Figure 1.

Figure 2. Concentration (pg/gm) of sex steroids in eggs at 1 hour postfertilization from control rainbow trout from experiments 1 and 2 for testosterone (T), 11-ketotestosterone (KT), 17β-estradiol (E2), and androstenedione (A). Each value represents the mean ± SE for a sample size of 30.

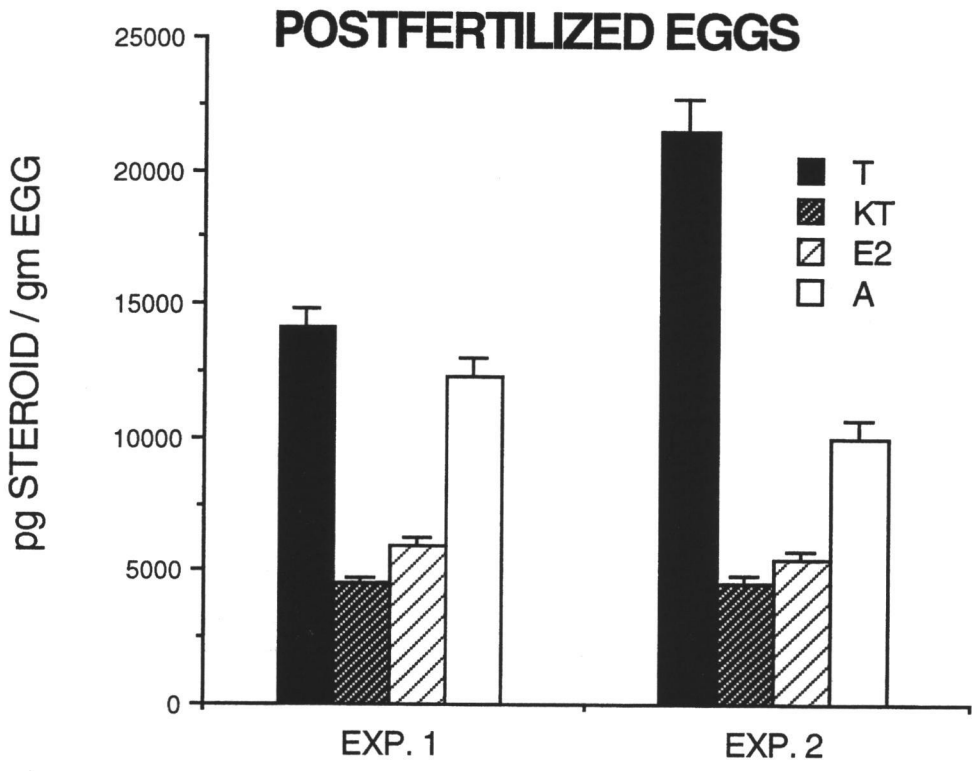
POSTFERTILIZED EGGS
pg STEROID / gm EGG
25000
20000
15000
10000
5000
0
T
KT
E2
A
EXP. 1
EXP. 2

Figure 2.

Figure 3. Concentrations (pg/gm) of sex steroids in control, female, and male groups of rainbow trout eggs at 1 hour postfertilization (0), and developing embryos at 5, 10,15, and 20 days postfertilization from experiment 1. Steroid designations as in Fig. 2. Each value represents the mean $\pm$ SE for a sample size of 30.
a and b=statistcally different from controls or males for the Mann-Whitney test for $P<0.05$.

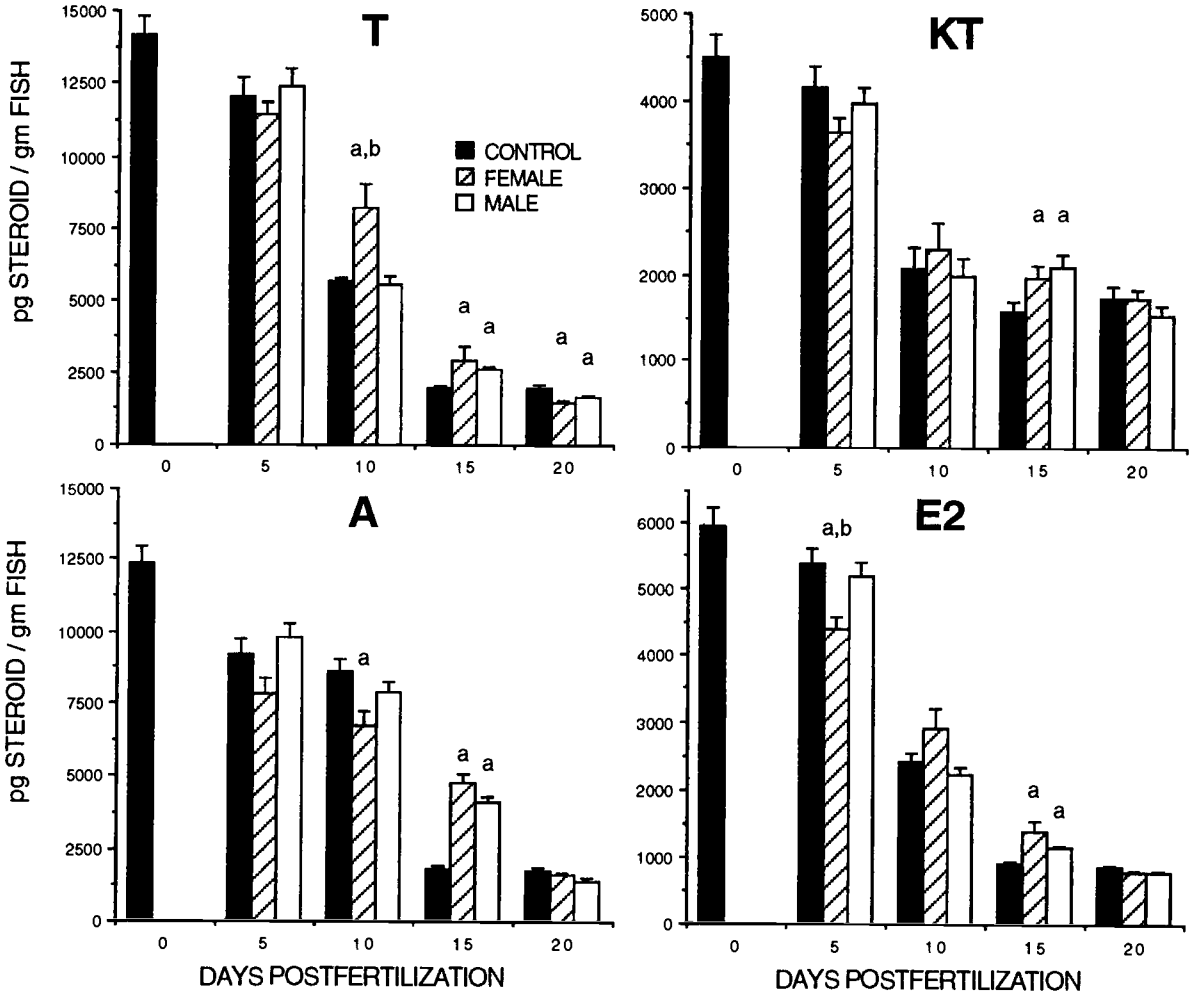
T
KT
A
E2
CONTROL
FEMALE
MALE
pg STEROID / gm FISH
pg STEROID / gm FISH
DAYS POSTFERTILIZATION
DAYS POSTFERTILIZATION
a,b
a
a
a
a
a
a
a
a
a
a
a
a,b
a
a
a
a

Figure 3.

DPF and A at 10 dpf and higher levels of T at 10 dpf. Both males and females had higher levels of all four steroids examined at 15 dpf. Females and males also had lower levels of T than controls at 20 dpf.

Hatch, Experiment 1.

Steroid profiles of rainbow trout from 20-67 dpf are given in Fig. 4. Except for T in males, steroid content increased around the time of hatch (32 dpf). A and KT increased at 25 dpf whereas T and E2 increased at 32 dpf. Steroid content differed between males and females at 25 dpf when males had higher levels of T, A, and E2. Monosex groups also differed from controls: females had lower levels of A at 25 dpf and males had higher levels of E2. At 32 dpf, both males and females had lower levels of T and A and higher levels of E2.

Yolk sac absorption, Experiment 1.

Steroid profiles of rainbow trout during yolk sac absorption (39 and 46 dpf) are given in Fig. 4. There was a general decrease in steroid content for all groups following hatch. There were differences between males and females at 39 dpf, when males had less T, A, and E2, and at 46 dpf, when males had less A, KT, and E2. Monosex groups also differed from controls at 39 and 46 dpf when males had less A and females had more E2. Males also had less T than controls at 46 dpf.

Figure 4. Concentrations (pg/gm) of sex steroids in control, female, and male groups of rainbow trout embryos from 20 to 67 days postfertilization from experiment 1. Steroid designations as in Fig. 2. Each value represents the mean $\pm$ SE for a sample size of 30.
a and b=statistcally different from controls or males for the Mann-Whitney test for $P<0.05$.

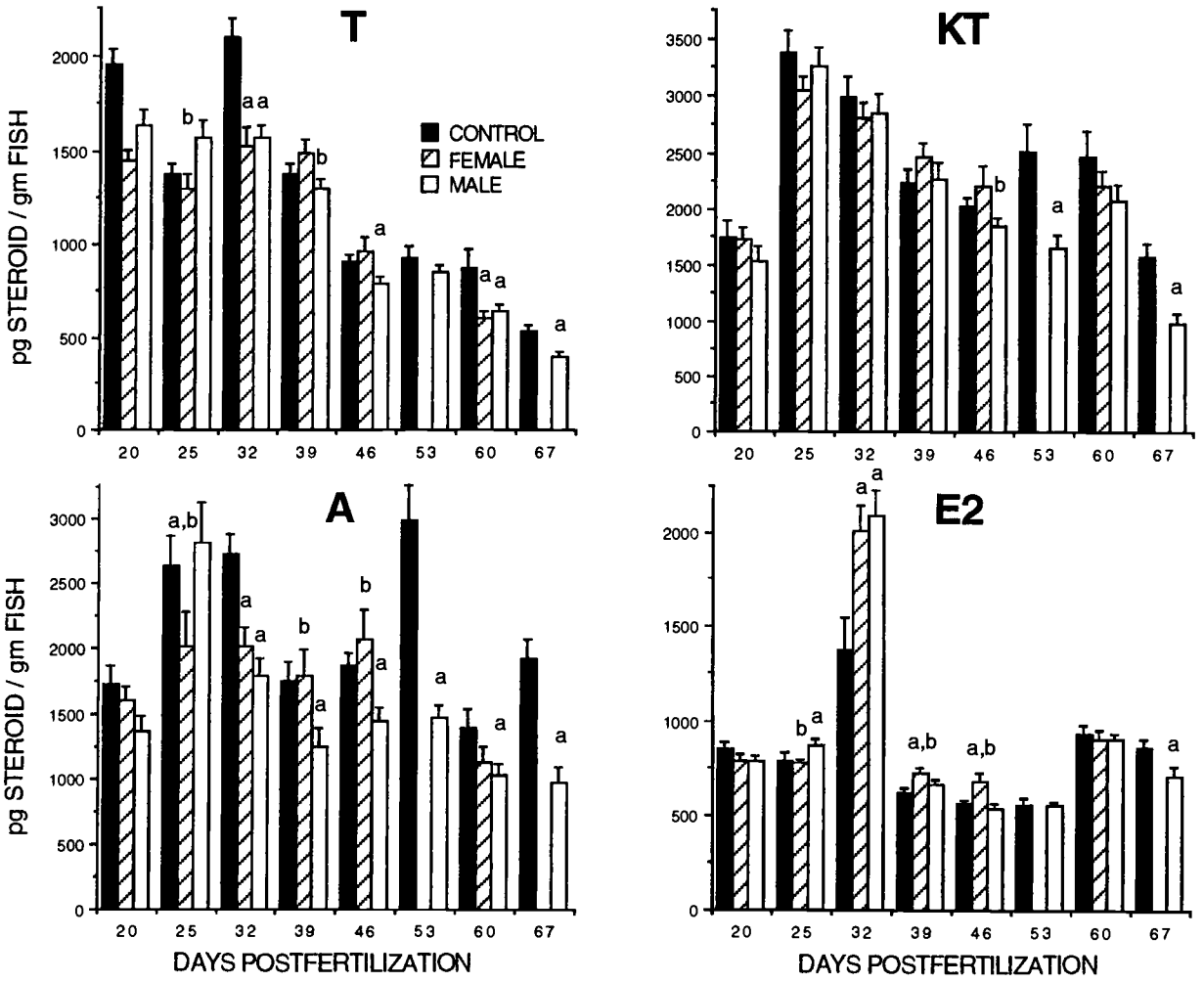
T
KT
A
E2
CONTROL
FEMALE
MALE
pg STEROID / gm FISH
pg STEROID / gm FISH
DAYS POSTFERTILIZATION
DAYS POSTFERTILIZATION
0
500
1000
1500
2000
2500
3000
3500
20
25
32
39
46
53
60
67

Figure 4.

Onset of feeding Experiment 1.

All steroids following the onset of feeding (50 dpf) were relatively constant or showed a decrease (Fig. 4). This was not true for A in control groups where levels fluctuated. There were no differences between monosex groups. Females contained less T than controls at 60 dpf and males contained less than controls for: T at 60 and 67 dpf, KT at 53 and 67 dpf, A at 53, 60, and 67 dpf, and E2 at 67 dpf.

G2 analysis, Experiment 1.

G2 analysis of steroid distributions are shown in Figs. 5 and 6. Values for g1 and g2 from postfertilized eggs to 67 dpf indicated that steroids among the three groups were not normally distributed throughout much of early development. Although g2 values were not statistically different from each other through time, non-normal distributions, as indicated by negative g2 values, became evident from eyed eggs to 67 dpf. No coherent patterns of steroid distributions, in terms of bimodality or unimodality between the groups of fish, were seen throughout the study period.

Histology, Experiment 1.

Rainbow trout showed the presence of undifferentiated gonads from hatching to 67 dpf. Undifferentiated gonads consisted of an aggregation of primordial germ cells on the germinal ridge. Some gonads appeared as a cyst-like

Figure 5. Kurtosis tests (g2/standard error) of sex steroids (pg/gm tissue) for control, female, and male groups of rainbow trout from 0-25 days postfertilization from experiment 1. Steroid designations as in Fig. 2.

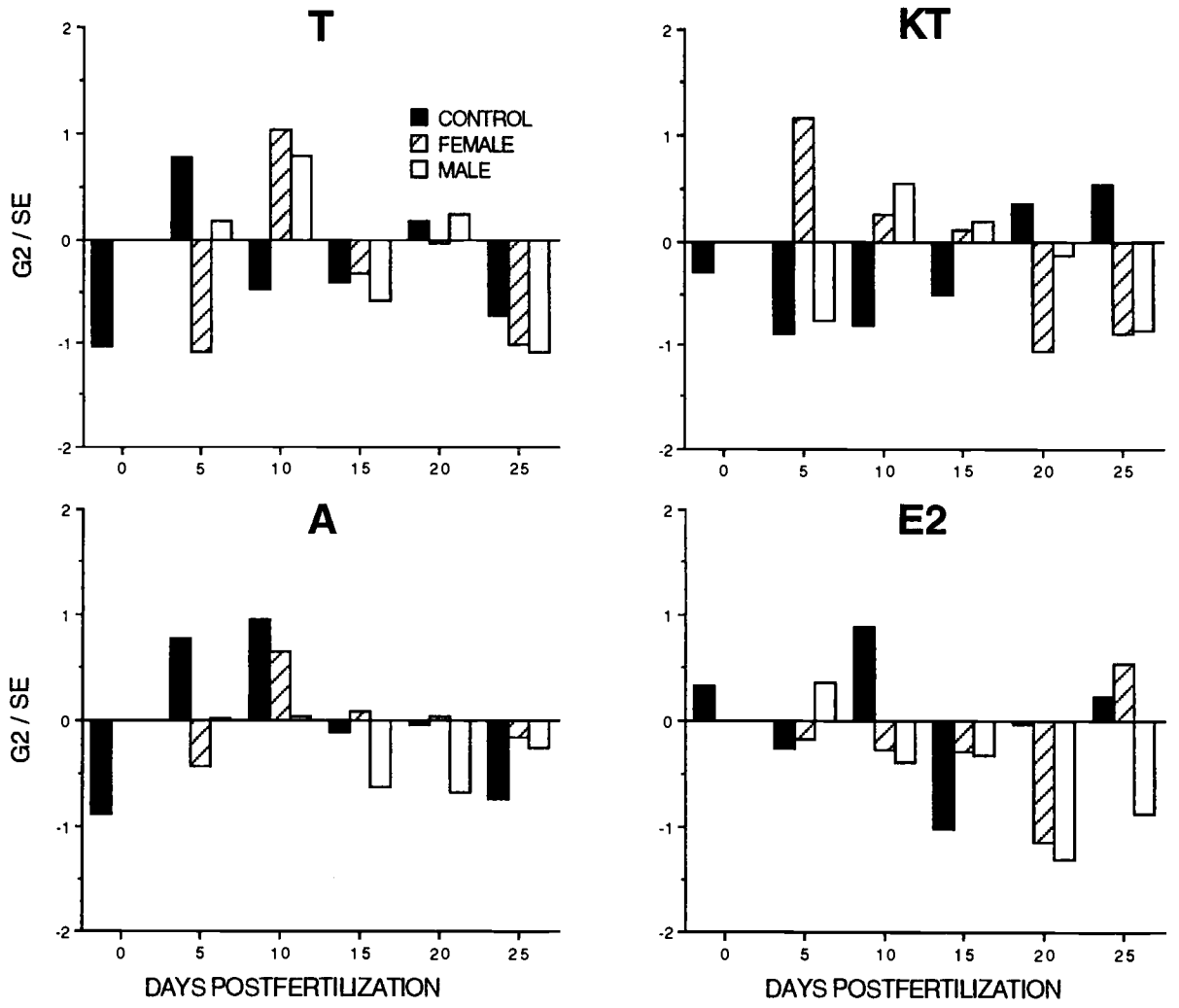
T
KT
A
E2
CONTROL
FEMALE
MALE
G2 / SE
G2 / SE
2
1
0
-1
-2
0
5
10
15
20
25
DAYS POSTFERTILIZATION
DAYS POSTFERTILIZATION

Figure 5.

Figure 6. Kurtosis tests (g2/standard error) of sex steroids (pg/gm tissue) for control, female, and male groups of rainbow trout from 32-67 days postfertilization (dpf) from experiment 1. Steroid designations as in Fig. 2. Hatching occurred at 32 dpf.

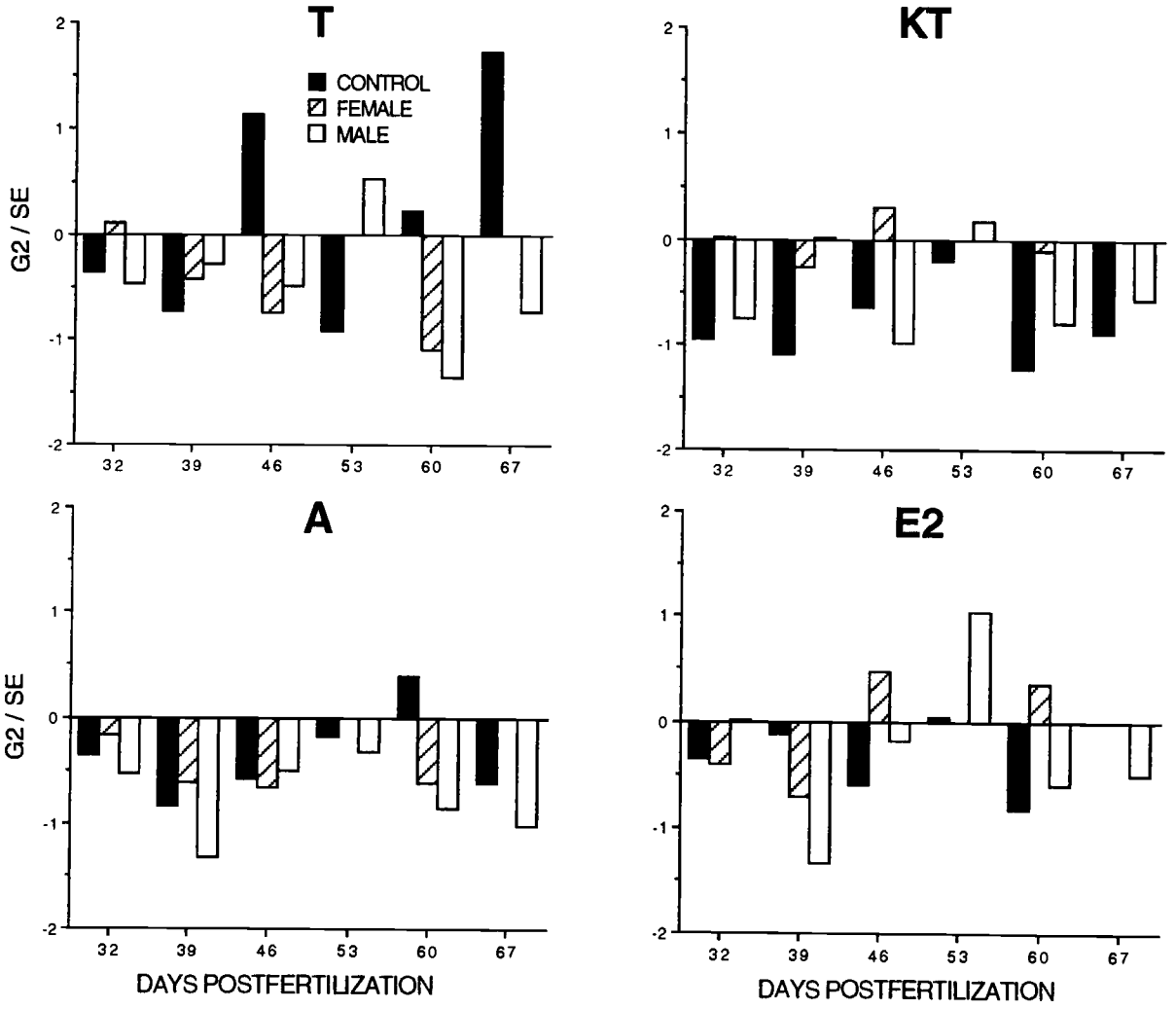
T
CONTROL
FEMALE
MALE
KT
A
E2
G2 / SE
G2 / SE
2
1
0
-1
-2
32
39
46
53
60
67
DAYS POSTFERTILIZATION
DAYS POSTFERTILIZATION

Figure 6.

structure but were still undifferentiated. It seems that fish from experiment 1 had not begun the process of gonadal differentiation by the end of sampling.

Post-fertilized eggs to 20 dpf, Experiment 2.

Steroid levels were relatively high in control eggs at 1 hr post-fertilization but declined precipitously by 20 dpf (Fig. 7). Steroids in male and female groups were first examined at 20 dpf. There were no differences in steroid content between males and females at this time. Monosex groups differed from controls at 20 dpf, males contained less T and both males and females contained less A.

Hatch, Experiment 2.

Steroid profiles of rainbow trout at hatch (48 dpf) are given in Fig. 8. There was an increase in steroid content around the time of hatch for all steroids. There were increases in T for all groups at 25, 30 and 48 dpf, KT for all groups at 30 dpf and for controls and males at 48 dpf, E2 for all groups at 48 dpf, and A for males at 25 dpf, for females at 30 dpf, and for controls at 48 dpf. There was a decrease in all steroids 30 days after hatch (78 dpf). There were differences between monosex groups for E2 at 48 dpf, males contained less than females, for A at 25 dpf, males contained more than females, and for A at 30 and 48 dpf, males contained less than females. Monosex groups differed from controls: for T at 25 dpf, females contained less, and

Figure 7. Concentrations (pg/gm) of sex steroids in control, female, and male groups of rainbow trout eggs at 1 hour postfertilization (0), and developing embryos at 12, 15, and 20 days postfertilization from experiment 2. Steroid designations as in Fig. 2. Each value represents the mean $\pm$ SE for a sample size of 30.
a=statistcally different from controls for the Mann-Whitney test for $P<0.05$.

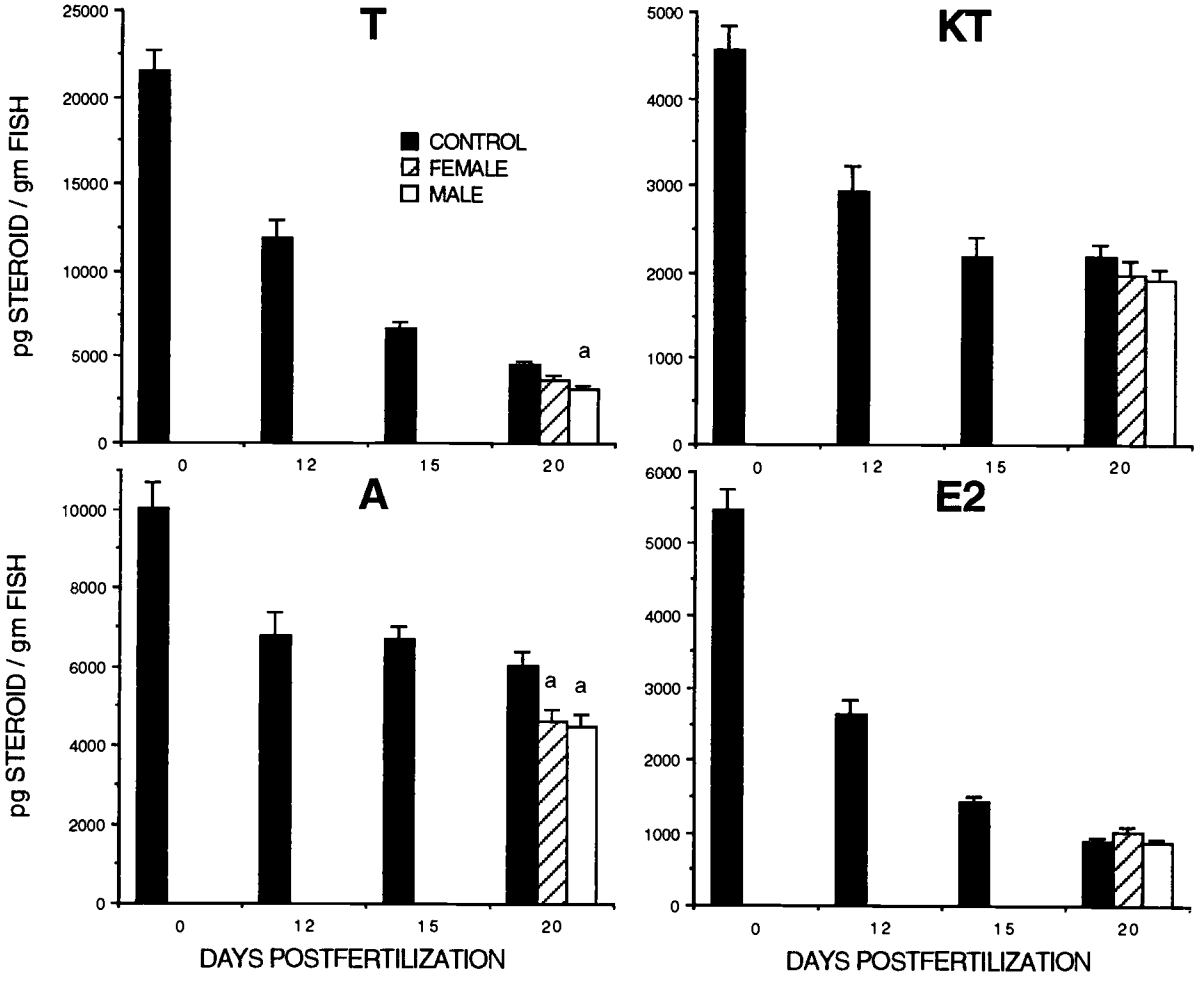
T
KT
A
E2
CONTROL
FEMALE
MALE
pg STEROID / gm FISH
pg STEROID / gm FISH
DAYS POSTFERTILIZATION
DAYS POSTFERTILIZATION
a
a
a
0
12
15
20
25000
20000
15000
10000
5000
4000
3000
2000
1000
8000
6000

Figure 7.

Figure 8. Concentrations (pg/gm) of sex steroids in control, female, and male groups of rainbow trout embryos from 20 to 78 days postfertilization from experiment 2. Steroid designations as in Fig. 2. Each value represents the mean $\pm$ SE for a sample size of 30. Hatching occurred at 48 dpf . a and b=statistcally different from controls or males for the Mann-Whitney test for $P<0.05$.

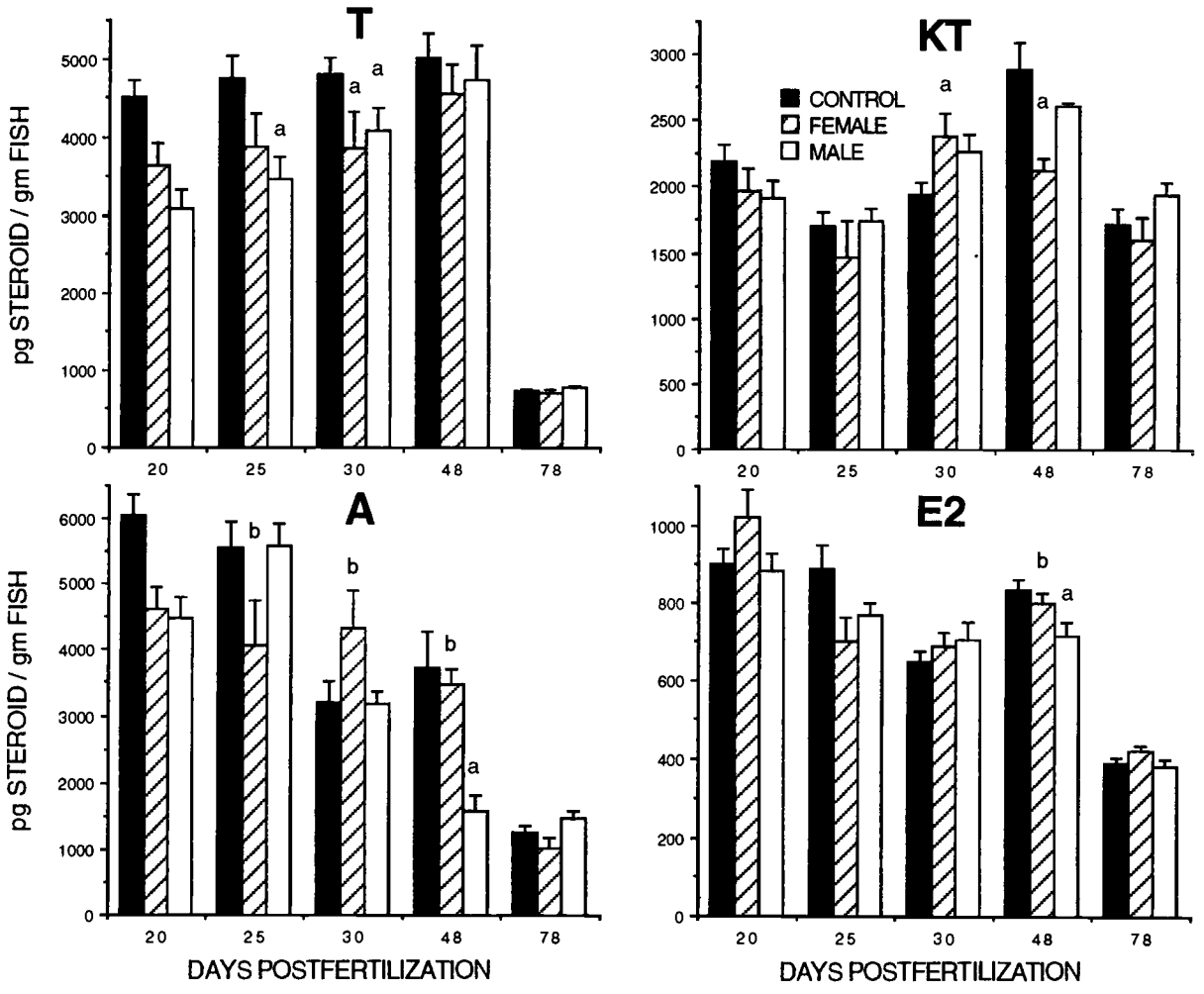
T
KT
A
E2
CONTROL
FEMALE
MALE
pg STEROID / gm FISH
pg STEROID / gm FISH
DAYS POSTFERTILIZATION
DAYS POSTFERTILIZATION
20
25
30
48
78

Figure 8

at 30 dpf, both females and males contained less, for KT at 30 dpf, males contained more, for KT at 48 dpf, males contained less, for A at 48 dpf, males contained less, and for E2 at 48 dpf, males contained less.

78-126 dpf, Experiment 2.

Steroid levels for rainbow trout at further developmental stages than those examined in experiment 1 are given in Fig. 9. Steroid declined from hatch until 78 dpf. There was a possible sexual dimorphism for T at 78, 90, and 100 dpf, levels were higher in males, lower in females, and intermediate in controls. There were also possible sexual dimorphisms for KT at 78 and 100 dpf, A at 78 dpf, and E2 at 78 and 90 dpf. Steroids in male and female groups also differed for T and A at 111 dpf, males contained more than females and for T, KT, and E2 at 126 dpf, males contained less than females. Monosex groups differed from controls at 100 dpf, females contained less A and more E2. At 111 dpf, both male and female groups contained less T, KT, and A than controls. Males also contained less E2 at 111 dpf. At 126 dpf, males and females contained less of all four steroids.

G2 analysis, Experiment 2.

G2 analysis of steroid distributions are shown in Figs. 10 and 11. Values for g1 and g2 from postfertilized eggs to 126 dpf indicated that steroids among the three groups were not normally distributed throughout much of early

Figure 9. Concentrations (pg/gm) of sex steroids in control, female, and male groups of rainbow trout embryos from 78 to 126 days postfertilization from experiment 2. Steroid designations as in Fig. 2. Each value represents the mean $\pm$ SE for a sample size of 30. Hatching occurred at 48 dpf. a and b=statistcally different from controls or males for the Mann-Whitney test for $P<0.05$.

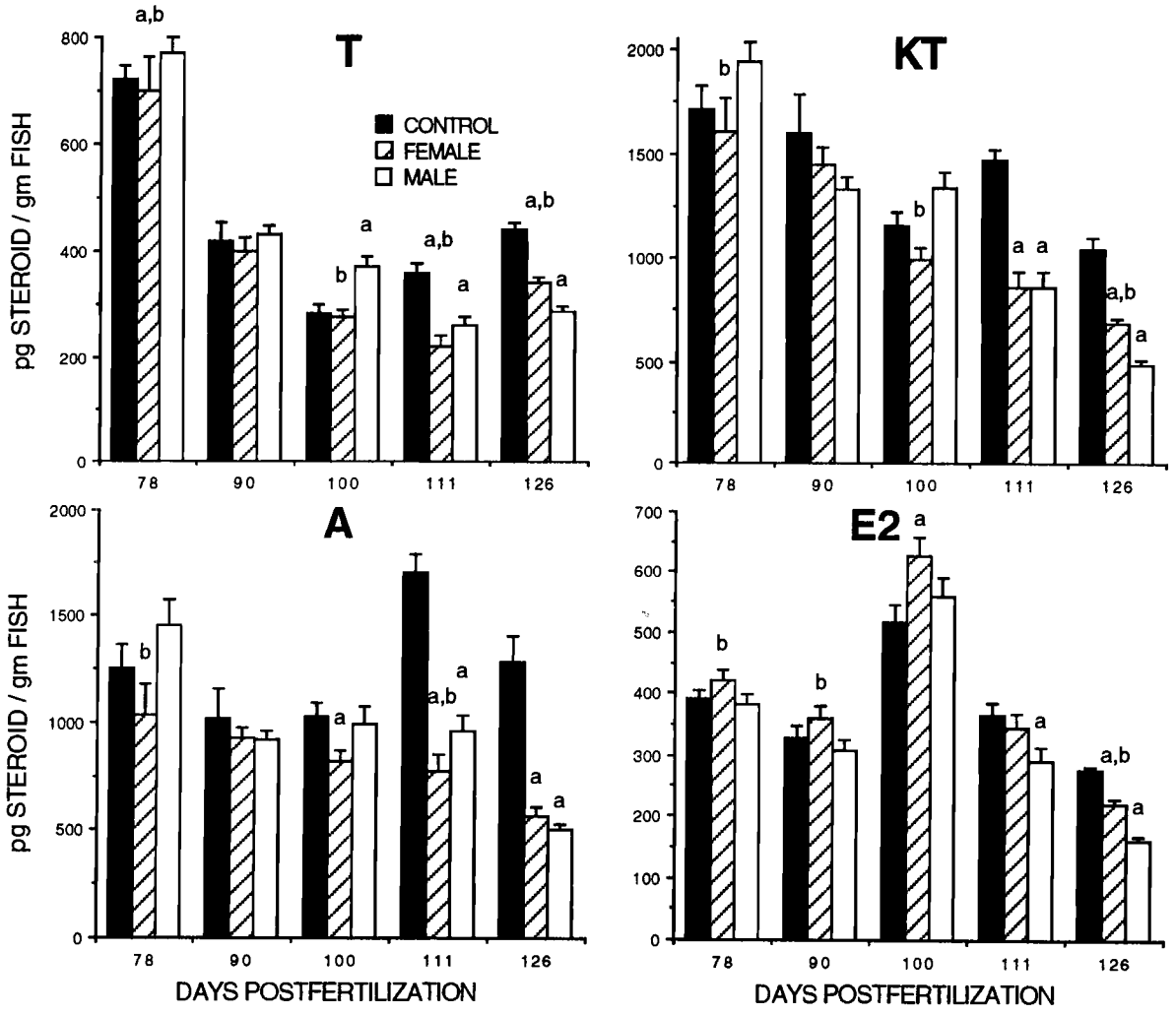
T
KT
A
E2
CONTROL
FEMALE
MALE
pg STEROID / gm FISH
DAYS POSTFERTILIZATION
78
90
100
111
126

Figure 9.

Figure 10. Kurtosis tests (g2/standard error) of sex steroids (pg/gm tissue) for control, female, and male groups of rainbow trout from 0-30 days postfertilization from experiment 2. Steroid designations as in Fig. 2.

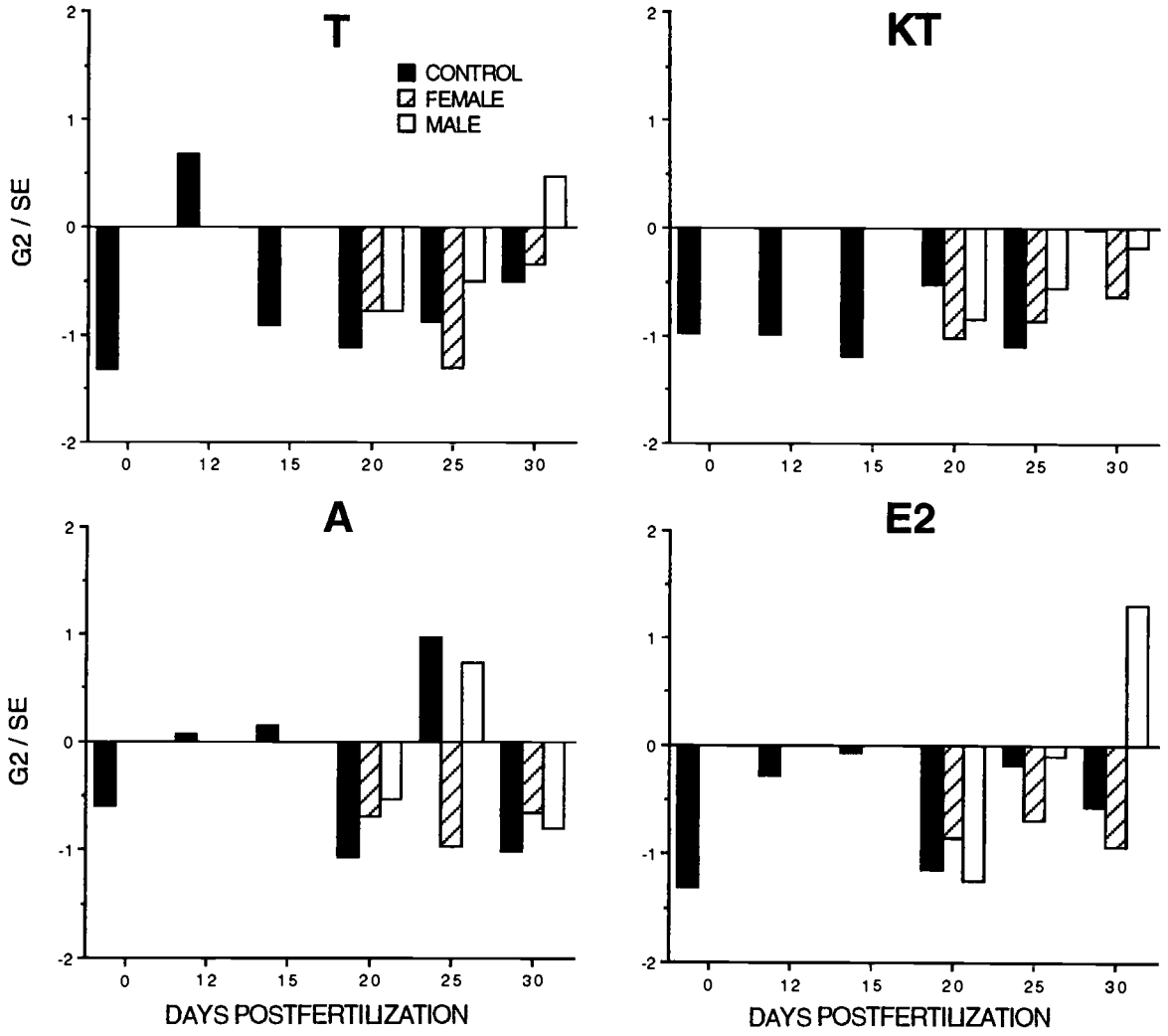
T
CONTROL
FEMALE
MALE
KT
A
E2
G2 / SE
G2 / SE
2
1
0
-1
-2
0
12
15
20
25
30
DAYS POSTFERTILIZATION
DAYS POSTFERTILIZATION

Figure 10.

Figure 11. Kurtosis tests (g2/standard error) of sex steroids (pg/gm tissue) for control, female, and male groups of rainbow trout from 48-126 days postfertilization (dpf) from experiment 2. Steroid designations as in Fig. 2. Hatching occurred at 48 dpf.

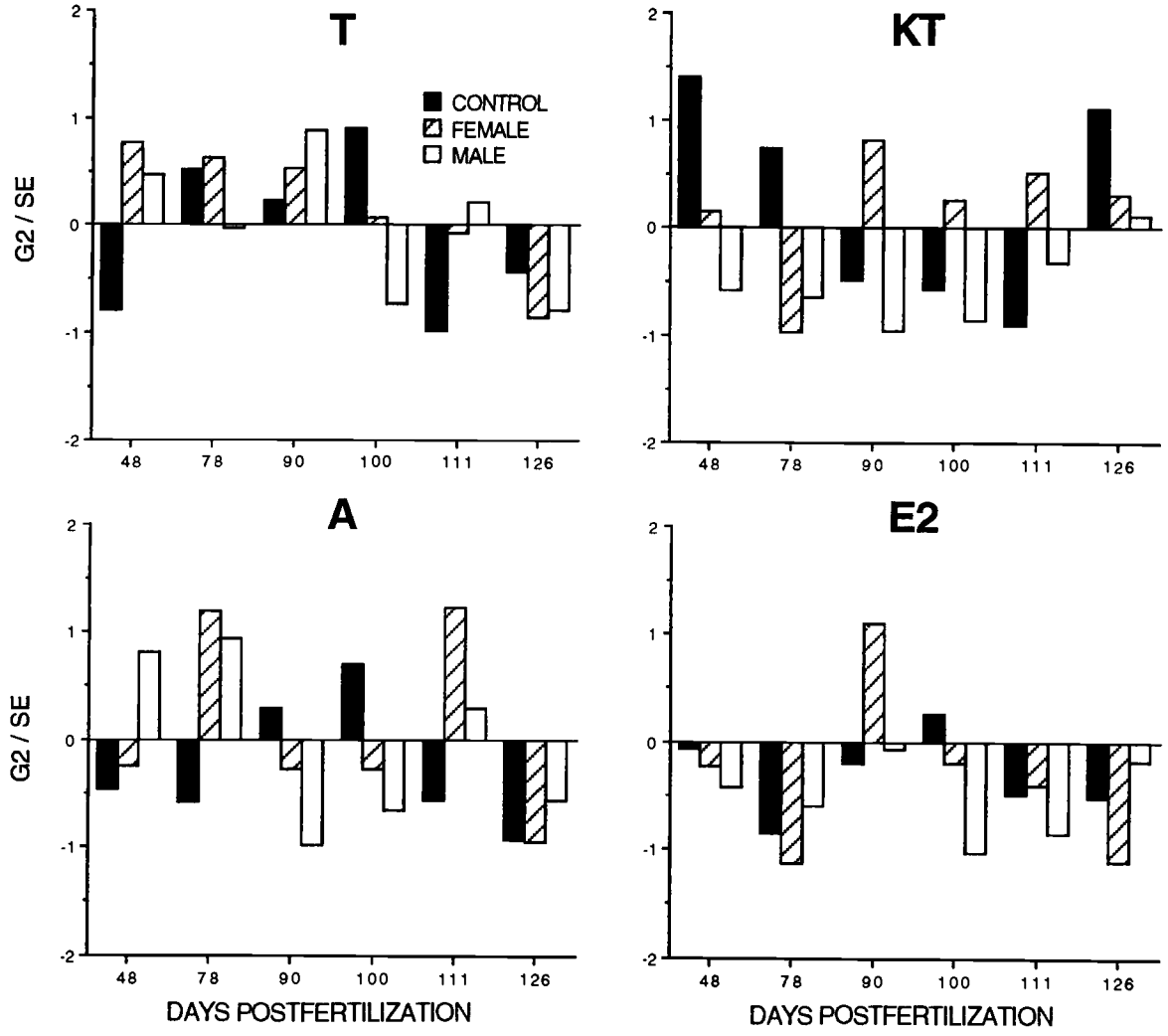
T
KT
A
E2
CONTROL
FEMALE
MALE
G2 / SE
G2 / SE
2
1
0
-1
-2
48
78
90
100
111
126
DAYS POSTFERTILIZATION
DAYS POSTFERTILIZATION

Figure 11.

development. Although g2 values were not statistically different from each other through time, non-normal distributions, as indicated by negative g2 values, were evident from postfertilized eggs to hatch. After hatch, g2 values became somewhat more positive for the androgens, while g2 value for E2 distributions remained negative. There were no coherent patterns of steroid distributions, in terms of bimodality or unimodality between the groups of fish, throughout the study period.

Histology, Experiment 2.

Rainbow trout contained undifferentiated gonads up to 78 dpf (Figs. 12 and 13). Undifferentiated gonads consisted of an aggregation of primordial germ cells on the germinal ridge. By 78 dpf some gonads appeared as a cyst-like structure but were still undifferentiated. By 90 dpf the first signs of differentiation had begun to appear. All female groups and roughly half the control samples showed the development of primary oocytes in either early meiosis or in the chromatin nucleolus stage. Some gonads at this stage showed slight signs of a lamellar structure. By 100 dpf presumptive ovaries contained larger numbers of oocytes in the chromatin nucleolus stage and showed further development of lamellar structures and compartmentalization of oocytes. By 111 dpf ovarian development could be clearly discerned based on the appearance of perinucleolar oocytes and a well defined lamellar structure.

Figure 12. Gonadal histology of gynogenetic rainbow trout from experiment 2 documenting differentiation. All sections are sagittal. (A) Undifferentiated gonad at 78 days postfertilization (dpf) showing a small number of primordial germ cells interspersed throughout the stroma. Magnification, 500X. (B) Gonad at 90 dpf showing compartmentalization of germ cells. Magnification, 640X. (C) Gonad at 100 dpf showing further compartmentalization of germ cells and slight lamellar structure. Magnification, 640X. (D) Developing ovary at 111 dpf with well defined lamellar structure. Magnification, 400X.

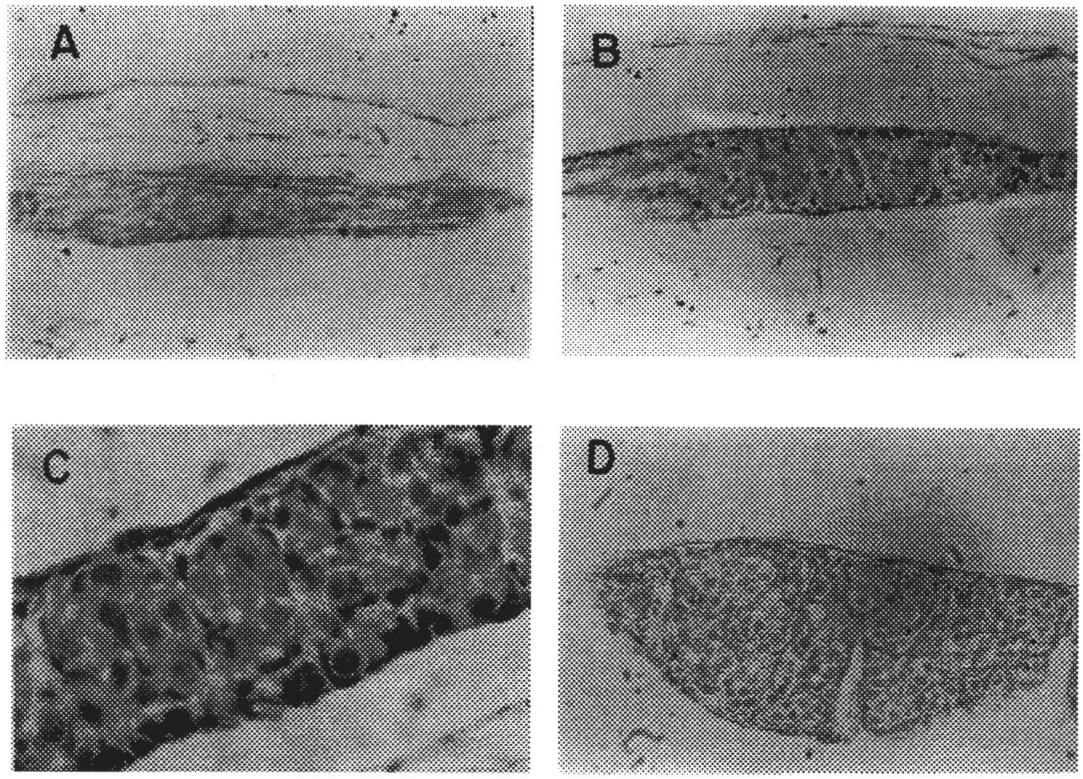

Figure 12.

Figure 13. Gonadal histology of androgenetic rainbow trout from experiment 2 documenting differentiation. All sections are sagittal. (A) Undifferentiated gonad at 78 days postfertilization (dpf) showing a small number of primordial germ cells interspersed throughout the stroma. Magnification, 500X. (B, C, D) Gonads at 90, 100, and 111 dpf showing proliferation of germ cells. Magnification, 640, 500, and 500X.

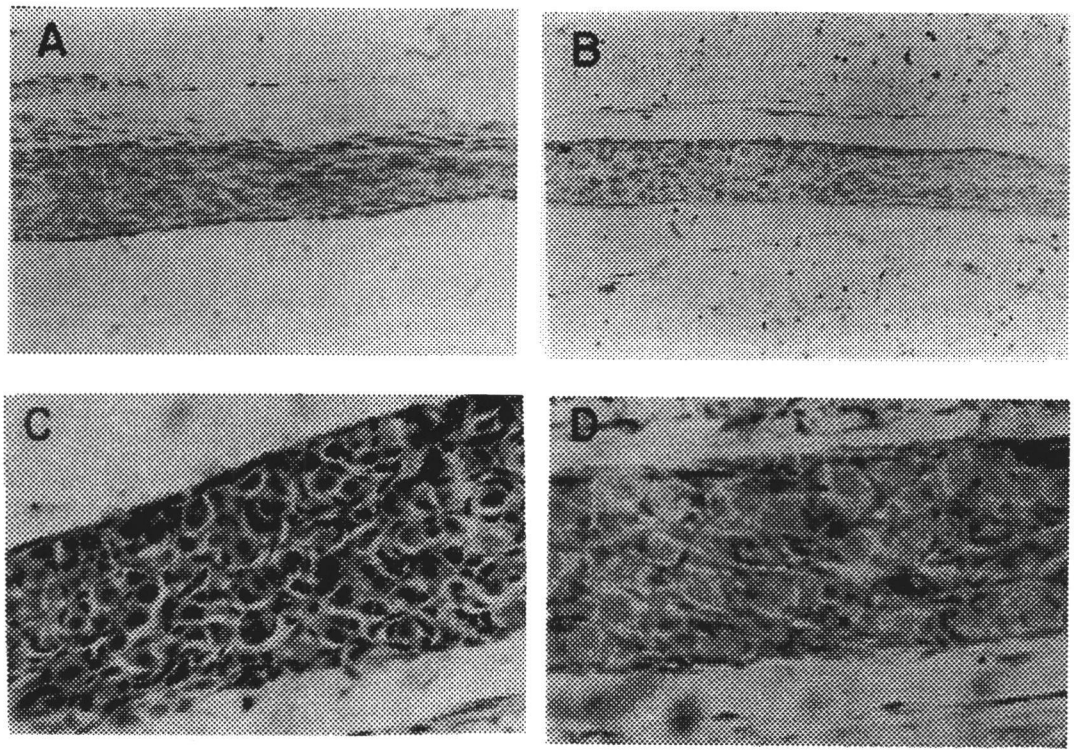

Figure 13.

Immunocytochemistry

Both immunoreactive FSH and GnRH were detectable in all three groups of fish at all dates examined (48-126 dpf). FSH was distributed exclusively in the ventral region of the proximal pars distalis (PPD). Sagittal sectioning of tissue revealed that FSH cells were concentrated in the lateral regions of the PPD (Fig. 14). GnRH was consistently found in the pituitary in the area where FSH was found and in the nucleus lateralis tuberis pars posterior (NLT) and inconsistently in the nucleus praeopticus periventricularis (NPP--Fig. 15) of the brain. Sagittal sectioning of the pituitary revealed a distribution pattern that was similar to that for FSH (Fig. 14). At hatch, only a few lightly staining cells immunoreactive for FSH or GnRH were observed. As development progressed, an increase in immunoreactivity for both peptide hormones was observed that was marked by a proliferation of darker staining cells. There were no differences between male, female, and control groups in the timing of the appearance of FSH and GnRH, localization, or intensity of staining. There was no staining when either normal rabbit serum, diluant, or primary antibodies that had been absorbed with purified antigen were used. LH was not detectable at any of the sampling dates (48-126 dpf) for any of the groups of fish.

To determine when FSH and GnRH first appeared during development, steelhead trout from the following brood year (1990) were examined at the eyed egg stage (19 dpf) and at

Figure 14. Sagittal sections through the brain and pituitary from rainbow trout documenting immunoreactivity for FSH and GnRH. (A) Pituitary from a male rainbow trout at 90 days showing light staining of FSH in ventral proximal pars distallis (ppd). Magnification, 400x. (B) Brain and pituitary from a female rainbow trout at 78 days postfertilization showing light staining of GnRH in ppd and nucleus lateralis tuberis (arrow). Magnification, 400x.

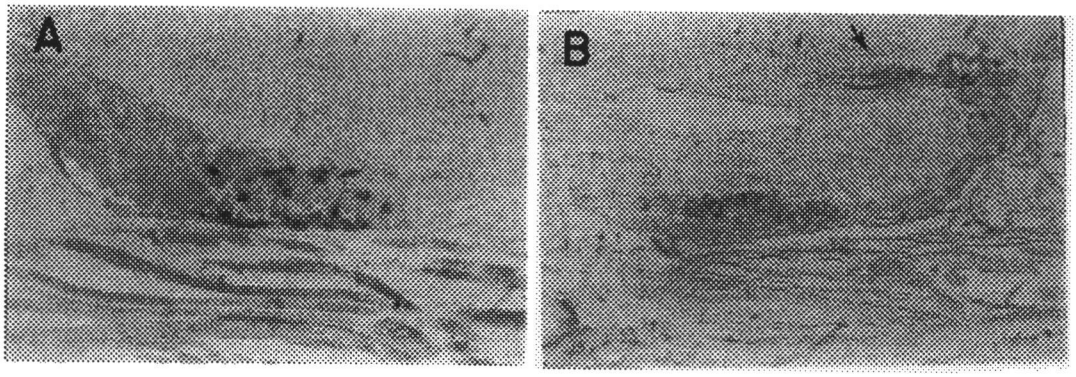

Figure 14.

Figure 15. Diagram of sagittal section through the brain and pituitary of a rainbow trout depicting sites of immunoreactive GnRH (•). C cerebellum; NLT nucleus lateralis tuberis; NPP nucleus praeopticus periventricularis; OL optic lobe; P pituitary; T telencephalon.

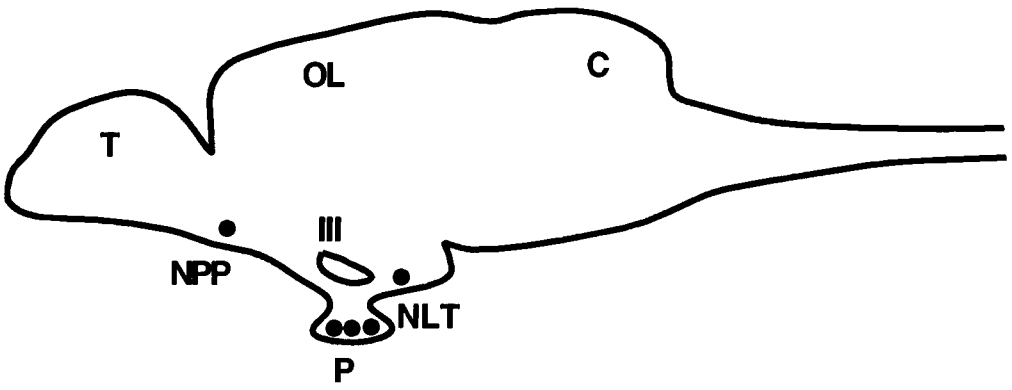

Figure 15.

hatch (30 dpf). There was no staining for either FSH or GnRH at 19 dpf whereas faint staining, similar to that during the previous year, was observed at hatch.

DISCUSSION

It seems that the brain-pituitary-gonadal axis in rainbow trout is intact at the time of hatch and during the process of gonadal differentiation. Given the dynamics of steroid hormone content during gonadal differentiation and the relative static nature of pituitary hormones, it seems that sex steroids may play a driving role for sexual differentiation of rainbow trout.

If the hypothesis that sex steroids are the natural inducers of sex differentiation in fishes is true (Yamamoto, 1969), one may expect to see changes in steroid levels in developing fish either before or during gonadal differentiation. We confirmed this for T, KT, A, and E2 in rainbow trout. The increase in whole body content of steroids at hatch and the possible dimorphic distributions around the time of gonadal differentiation may indicate a possible role of sex steroids in this process. Changes in steroid levels at hatching may also be indicative of the onset of sexual differentiation, even though no signs of gonadal differentiation were histologically discernible.

The lack of a coherent pattern of bimodal distributions of steroids in the control fish and unimodal distributions

in the monosex groups in the g2 statistic was problematic. Previous work with coho salmon (Feist *et al.*, 1990) revealed bimodal distributions of steroid content in fish populations of mixed sex around the time of hatching. Rothbard *et al.*, (1987) found that testosterone concentrations in 6 to 8 week old tilapia fry in mixed sex populations had bimodal distributions, whereas interspecific crosses, characterized by 100% male progenies, had unimodal distributions of testosterone content. The g2 statistic becomes less sensitive in sample sizes of less than 75. Relatively low levels of steroids in the fish, which approached the lower limits of the RIAs, may also have obscured differences if they existed. Monosex groups of fish also tended to grow faster than control animals after the onset of gonadal differentiation. This was especially apparent for the all male group. This may have led to lower levels of steroids in the monosex groups than in the controls during later periods of development. This discrepancy may have obscured the dimorphic differences in steroid content and the possible differences in the steroid distributions. Dimorphisms in steroid content at the time of gonadal differentiation, which were not observed at any other time during development, support a role of steroids in this process.

Developing rainbow trout were able to synthesize T, KT, A, and E2 before the onset of gonadal differentiation, and patterns of hormonal concentrations possibly distinguished themselves dimorphically around the time when gonadal

differentiation could be identified with light microscopy. Steroids were relatively high in postfertilized eggs, decreased markedly during the period before hatch, and next increased slightly at the time of hatching. Hormone levels remained relatively constant or decreased after yolk sac absorption for all examined steroids. Histological analyses revealed the presence of an undifferentiated gonad up to 78 dpf, followed by ovarian development about 12 days later. Immunoreactive FSH and GnRH but not LH were observed at all sampling dates. There were no differences between males and females.

Although few investigators examined the onset of steroidogenesis in the differentiating teleostean gonad, most provided evidence that supports our finding of steroidogenic capabilities of rainbow trout during very early developmental stages. Early steroidogenic capabilities were documented for the guppy (Takahashi and Iwasaki, 1973), rainbow trout (van den Hurk *et al.*, 1982; Antila, 1984; Fitzpatrick *et al.*, 1993; Yeoh *et al.*, 1996a), and tilapia (Rothbard *et al.*, 1987).

Observations that exogenous steroids can influence gonadal development support the role of sex steroids as possible inducers of sexual differentiation in fish. The ability of exogenous androgens to influence gonadal development in a male direction and feminization by administration of estrogenic compounds has been documented for a wide variety of salmonids (e.g. Goetz *et al.*, 1979;

Redding *et al.*, 1987; van den Hurk and Slof 1981; van den Hurk and van Oordt, 1985; Piferrer and Donaldson, 1989, 1991; Feist *et al.*, 1995). Steroids are usually administered at the onset of feeding, which coincides with the period when we found the first signs of gonadal differentiation and possible dimorphisms in sex steroid content. Sex reversal can also be accomplished by immersing embryos in steroids around the time of hatch. The increase in steroids seen at hatch in this study may be playing a role similar to that of exogenously administered hormones.

The finding that steroids were relatively high in postfertilized eggs is not surprising. Steroids are lipophilic and easily cross cell membranes. Previous work revealed that the steroid profile in ovarian fluid of coho salmon is paralleled by unfertilized eggs (Feist *et al.*, 1990). Yeoh *et al.* (1996b) also found relatively high levels of T, KT, and E2 in postfertilized eggs of steelhead trout. Sex steroid content of postfertilized eggs was similar to that in plasma of ovulating coho salmon (Fitzpatrick, 1986). We can only speculate about a possible role for these steroids in either gametes or the early developing embryo.

The precipitous decline in T, KT, and E2 content of developing embryos from unfertilized eggs to 20 dpf was also documented in tilapia (Rothbard *et al.* 1987), coho salmon (Feist *et al.*, 1990), and steelhead trout (Yeoh *et al.*, 1996b). Antila (1984) showed that rainbow trout embryos metabolized steroids at 0 to 8 dpf, and Yeoh *et al.* (1996a)

demonstrated this ability at 22 dpf. It is difficult to believe that the decline in steroids can be explained by their leaching out of the eggs, particularly when the lipophilic nature of these molecules is considered. However, Yeoh *et al.* (1996a,b) found that steelhead trout embryos were able to form steroid glucoronides during very early life stages. This may be a mechanism by which embryos render steroids water soluble to excrete them. The decline in steroid content during the period before hatch is therefore most likely due to metabolic processing of maternal steroids by embryos.

Increases in steroid content at hatch in this study were also observed in tilapia (Rothbard *et al.*, 1987), coho salmon (Feist *et al.*, 1990), and steelhead trout (Yeoh *et al.*, 1996b). The increase in steroidal content at hatch is most likely due to synthesis by embryos but may also be caused by metabolic processing by embryos of other maternal steroids not monitored by RIA.

Fish embryos are sensitive to the sex reversing properties of exogenous steroids around the time of hatching (Piferrer and Donaldson, 1989, 1991; Feist *et al.*, 1995). Steroid dynamics at the time of hatch may be indicative of the onset of sexual differentiation before this event is histologically discernible. It is possible that a relatively small increase in steroids at hatch may affect or be involved in sexual differentiation after embryos have been exposed to extremely high levels of maternal hormones during

early development.

Possible sexual dimorphisms in steroid content before and during histological signs of gonadal differentiation in this study have been documented elsewhere. Sexual dimorphisms for T have been shown in tilapia during very early development (Rothbard *et al.* 1987), for T, KT, and A in presumptively sexed coho salmon at 101 dpf (Feist *et al.*, 1990) and in steroid production by gonads and interrenals of rainbow trout shortly after the onset of gonadal differentiation (Fitzpatrick *et al.*, 1993). Differences in KT content between all female and mixed sex groups of steelhead trout during early development has also been documented (Yeoh *et al.*, 1996b). Sexual dimorphisms in steroid levels during later development have also been seen in the plasma of coho salmon, as during smoltification (Patino and Schreck, 1986), and final maturation (Fitzpatrick *et al.*, 1986).

Immunocytochemical localization of GnRH is in general agreement with Crim *et al.*, (1979), Halpern-Sebold and Schreibman (1983), and Kah *et al.*, (1984) who found distributions of this hormone in similar areas of the brain in neonatal lamprey and platyfish and adult goldfish, respectively. Immunocytochemical localization of FSH is in agreement with van den Hurk (1982), Mal *et al.*, (1989), and Nozaki *et al.*, (1990a) who found similar areas of staining in the pituitary during early development for rainbow trout and coho salmon. The appearance of FSH immunoreactivity

around the time of hatch was also noted by Mal *et al.* (1989). The lack of specific LH immunoreactivity in rainbow trout at 126 dpf is not surprising given that this gonadotropin is considered to be maturational and has not been found before puberty in coho salmon (Mal *et al.*, 1989; Nozaki *et al.*, 1990b).

Our histological analyses of developing gonads are in general agreement with those reported for rainbow trout by Takashima *et al.* (1980) and van den Hurk and Slof (1981), and for other salmonids by Robertson (1953), Ashby (1957), and Goetz *et al.* (1979). At this level of resolution gonadal differentiation appears to occur in a 1 week period about 40 days after hatch. Up to this point, no clear distinctions could be discerned between males and females. The inability to identify sex before this period is problematic for research on sexual differentiation. Our histological analysis for the monosex populations of rainbow trout did not demonstrate subtle differences in gonadal differentiation between the sexes before that time. The appearance of meiotic figures in presumptive ovaries remains to be the first sign of gonadal differentiation observable at the light microscopy level. Correlation of hormonal events with histology of the differentiating gonad for monosex populations provided useful information on the possible influence of sex steroids during sexual differentiation.

Although we have shown that the steroidal milieu of

developing rainbow trout is indeed dynamic during the period of sexual differentiation, it is still impossible to conclusively determine whether sex steroids are a cause of sexual differentiation or simply a result of it. The lack of coherent patterns in steroid distributions during early development compounds this problem. The possible differences in steroid content between monosex groups slightly before gonadal differentiation was histologically discernible adds strength to the argument that steroids are influencing gonadal development, yet signs that this process has begun may not be detectable at our level of resolution. The further use of monosex populations and a more complete description of the development of the brain-pituitary-gonadal axis during early life stages may increase our understanding of this process.

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III. THE PRODUCTION OF FUNCTIONAL SEX-REVERSED MALE RAINBOW TROUT WITH 17α-METHYLTESTOSTERONE AND 11β-HYDROXYANDROSTENEDIONE. [1]

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[3] Supported jointly by the Oregon State University, Oregon Department of Fish and Wildlife, and the U.S. Geological Survey.

ABSTRACT

The sex of gynogenetic rainbow trout was reversed to produce XX males by using two steroids, 17α-methyltestosterone (MT) and 11β-hydroxyandrostenedione (OHA). Fish were exposed to either single or multiple doses of steroids during various times around the period of hatching to determine the labile period for effective sex reversal. Steroids were administered either by immersion (400 µg/ l for 2 hr) or a combination of immersion plus feeding (3 mg/kg diet for 60 days) to determine if males with intact sperm ducts could be produced. Immersion in MT resulted in varying degrees of masculinization while immersion plus feeding produced nearly 100% male populations. The most effective period for steroid immersion was one week past the time when one half of the fish had hatched. Multiple immersions in MT failed to increase masculinizing effects. Immersion in OHA caused only low rates of masculinization, while immersion plus feeding resulted in 70% male populations. Males produced through both immersion and feeding of MT generally did not develop sperm ducts; whereas animals treated by immersion alone in MT, or those produced with OHA, tended to be functional. Although final yields for sex-reversed males following gynogenesis was low, 1.5 and 1.6% for the two groups respectively, the use of cryopreserved semen from these males can be used to produce all female offspring for years.

Cryopreserved semen from functional males in this study produced 100% female populations.

INTRODUCTION

The use of masculinized female salmonids (XX males) to produce all-female populations is now a well-documented method for control of sex in aquaculture systems (Bye and Lincoln, 1981; Hunter *et al.* 1982, Hunter and Donaldson, 1983). Masculinization usually involves the use of the synthetic androgen 17α-methyltestosterone (MT) fed at a dose of 3 mg/kg diet for 60 days beginning with the onset of feeding. Although this procedure typically results in populations of up to 100% males, the majority of the animals lack or have incomplete sperm ducts and semen must be removed surgically (Bye and Lincoln, 1981). This procedure is both time consuming and detrimental to broodstock. Treating normal populations of fish with MT also requires waiting until the animals are sexually mature to determine through breeding experiments or gonadal morphology which are XX males. The use of chromosomally manipulated all-female (gynogenetic) fish ensures that all sex-reversed animals will be XX males.

Sex reversal can also be accomplished by immersing the fish in steroids during early development. Masculinization or feminization can be accomplished with a single immersion applied around the time of hatching (Piferrer and Donaldson, 1991, 1992). The procedure is both time-and cost-effective and 90-100% of the fish are sex-reversed.

Masculinization by immersion usually employs the use of MT. van den Hurk and van Oordt (1985), however, found that the naturally occurring steroid, 11β-hydroxyandrostenedione (OHA), resulted in transient masculinization of rainbow trout (*Oncorhynchus mykiss*) when fish were either immersed in or fed the steroid. They also found that feeding OHA had a longer lasting masculinization effect. Redding *et al.* (1987) reproduced these findings with coho salmon (*O. kisutch*), with the exception that fish fed OHA were permanently masculinized

This study was designed to determine whether a gynogenetic group of rainbow trout could be sex-reversed to males with intact sperm ducts by using MT or OHA. The study also sought to determine when fish were most sensitive to the masculinizing effects of the androgens, whether multiple doses resulted in greater masculinization, and if masculinized females were capable of producing 100% female offspring.

MATERIALS AND METHODS

Gynogenesis and fish rearing

Eggs from 19 females and semen from 5 males were obtained from Oregon State University's Department of Food Science and Technology in December 1989 and transported on ice to the Fish Genetics and Performance Laboratory at Smith Farm, Corvallis. Gynogenesis was performed following a

method reported by Thorgaard *et al.* (1983). Briefly, pooled semen (1 ml) was diluted 1:10 with 0.3 M glucose, poured into 16 x 25.5 cm glass trays, and irradiated with ultraviolet light at an intensity of 400 $\mu W/cm^2$ for 2 min to inactivate the sperm. Pooled eggs (about 3000) were poured directly into the trays containing inactivated sperm, mixed with water, and held for 2 min, then rinsed and held for an additional 8 min. One half of the seven lots of fertilized eggs (21,000) were then heat shocked at 29°C for 10 min in a circulating water bath, and the other half at 26°C for 20 min. Normal, mixed sex controls (3000) were produced in the same manner except that semen was not irradiated and the eggs were not heat shocked. The resulting fry were raised under a normal photoperiod and at a water temperature of 11.5-13.5°C.

Sex reversal

All female groups created by gynogenesis were exposed to two steroids, 17α-methyltestosterone (29 °C heat shock group only) and 11β-hydroxyandrostenedione (26 °C heat shock group only). Steroids were applied through immersion as either single or multiple doses at various times near the period of hatching or by a combination of immersion plus feeding (see Table I).

Immersions were carried out following a method by Goetz *et al.* (1979). Embryos at 20 days post-fertilization (dpf) were divided into groups of 100 each for the MT immersions

and 125 each for the OHA immersions and placed in 14x14x5 cm plastic chambers fitted with 1-mm^2 plastic mesh top and bottom which was then placed into Heath trays (4 chambers/tray). The steroid immersions were carried out in 14 l buckets in which the plastic chambers were floated. Each bucket was well oxygenated with an airstone. Either MT or OHA were dissolved in ethanol (1 mg/ml) and 4 ml added to 10 l of well water at 11.5 $^{\circ}C$ to obtain a final concentration of 400 µg steroid /l. Embryos were immersed for 2 hours, rinsed, and then placed back in Heath trays. Groups were arranged in Heath trays to ensure that there was no contamination of other groups following treatments. All female control groups were immersed in 4 ml of ethanol/10 l of well water.

Hatching occurred at 30 dpf. Fish were transported from Heath trays to the 14 l flow-through tanks at 47 dpf and placed on Biomoist starter #1. Those fish placed on steroid diets were fed at 3 mg/kg diet for 8 weeks. Steroid diets were prepared by dissolving MT or OHA in ethanol and spraying over feed. Control diets consisted of feed that was sprayed with ethanol. The fish were fed 3 or 4 times daily to satiation with treated or control diets.

Sex was determined at 174 dpf by sampling fish (n=15-25) from each group and examining gross gonadal morphology under a dissecting microscope. Experimental groups not showing significant masculinization were discarded. At 2 years of age, fish from each group were checked for ripeness

by gentle pressure on the abdomen. Females with hand-strippable eggs were discarded. Males with the ability to extrude milt through the gonopore were tagged with passive integrated transponders for future identification, and semen samples were cryopreserved for future progeny testing. Sperm motility was checked under a microscope. All remaining animals were sexed by visual examination of gonads.

Cryopreservation of semen

Semen from XX males was cryopreserved because no eggs were available at the time when males became ripe. Cryopreservation of semen was conducted following a method by Wheeler and Thorgaard (1991). Semen was mixed with a cooled (4 $^{\circ}$C) extender solution composed of 5.4% glucose, 9.0% DMSO and 10.0% fresh hen's yolk (Alderson and MacNeil, 1984; Scheerer and Thorgaard, 1989) in a ratio of 1 part semen to 3 parts extender. One ml of this mixture was put into a 1.7 ml microfuge tube and placed directly on crushed dry ice for 5 min before being placed in liquid nitrogen.

Progeny testing

Eggs from 6 females and semen from 4 males were obtained from the Department of Food Science and Technology in June 1992 and transported on ice to the Fish Genetics and Performance Laboratory. After thawing the cryopreserved semen for 2.5 min at 13 $^{\circ}$C, 1 ml was added to 21 g (about 250) of pooled eggs. Semen from 26 XX males was used. Pooled

semen from normal males was used to produce control fish. Survival of resulting progeny was monitored to the onset of feeding. Sex of progeny was determined at 6 months of age by examining the gonads of 30-50 fish under a dissecting microscope.

Statistics

The Chi-square test was used for analysis of alterations in sex ratios. Intersex and sterile fish were combined with females in the groups treated with steroids to ensure that only completely sex-reversed fish were considered as having been affected by the treatment.

RESULTS

Gynogenesis

Heat shocks at either 26 or 29°C produced 100% female populations of rainbow trout. Two mixed sex control groups consisted of 56 and 52% females, respectively. Survivorship for the gynogenetic groups was low, but consistent with results from past experiments. Survival at the developmental stages of eyed eggs, hatch, onset of feeding, and final yield for the 29°C heat shock group was 8, 5, 3, and 1.5% respectively; survival for the 26°C heat shock group was 8, 7, 3, and 1.6%; whereas survival for the control group was 98, 97, 76 and 74%.

Sex reversal

Steroid immersion and feeding resulted in varying degrees of masculinization (Table I). MT was effective in sex reversing experimental groups when applied either as immersion only or in a combination of immersion plus feeding. After exposure to a single immersion treatment, MT produced the highest percentage of males in group 3 when applied at 1 week past the time when one half of the fish had hatched (50% hatch) and was least effective when applied 1 week before 50% hatch (group 1). Multiple immersions in MT decreased the number of males produced (groups 5-10). Nearly 100% males were produced in groups 11 and 12, and 100% were male (group 13) when fish were both immersed in and fed MT. OHA failed to effectively sex reverse rainbow trout when applied by immersion only (groups 14-21), but did have a masculinizing effect when both immersion and feeding were employed (groups 24-26). All experimental groups contained some sterile and intersex fish. Gonads of steroid treated fish ranged from mature females with strippable eggs to mature males with strippable milt (Fig. 16).

The percentage of mature males with intact sperm ducts, or which had the ability to extrude milt from the gonopore, are given in Table I. Because animals immersed in OHA only did not yield a high percentage of males, they were discarded after sexing. Fish either immersed in MT, or immersed in and fed OHA, tended to have a large percentage

Table I. Percent males, functional males, intersex, and sterile fish produced from gynogenetic rainbow trout treated with 17α-methyltestosterone (MT) or 11β-hydroxyandrostenedione (OHA).

Group no.	N	Steroid treatment	Week relative to hatch				Feed	% Males[1]	% Intersex	% Sterile	% Func. males
			-1	0	+1	+2					
1	25	MT	X					16[b]	0	4	100
2	19			X				68[c]	0	0	75
3	37				X			76[c]	3	0	92
4	26					X		62[c]	15	8	88
5	16		X	X				25[c]	0	0	100
6	32		X	X	X			53[c]	12	3	92
7	18		X	X	X	X		61[c]	0	0	86
8	28			X	X			46[c]	4	4	60
9	26			X	X	X		38[c]	8	8	100
10	20				X	X		30[c]	5	5	100
11	28			X	X	X	X	96[c]	0	0	0
12	17				X	X	X	94[c]	0	0	0
13	15					X	X	100[c]	0	0	40
14	15	OHA	X					13[b]	0	0	--
15	15			X				13[b]	0	0	--
16	15				X			0	0	0	--
17	15					X		13[b]	0	0	--
18	15		X	X				0	0	0	--
19	15		X	X	X			13[b]	0	0	--
20	15		X	X	X	X		7[a]	0	0	--
21	15			X	X			0	0	0	--
22	--			X	X	X		--	--	--	--
23	--				X	X		--	--	--	--
24	32			X	X	X	X	72[c]	9	6	75
25	35				X	X	X	69[c]	0	6	100
26	35					X	X	71[c]	6	9	100
27	28	Control ethanol	X					0	0	0	0
28	29	(all female group)	X	X	X			0	0	0	0
29	25	No treatment						44[c]	0	0	100
30	25	(mixed sex group)						48[c]	0	0	100
31	33	No treatment						0	0	0	0
32	30	(all female group)						0	0	0	0

Steroids were applied as either an immersion or immersion plus feeding. Each "X" represents the time and type of exposure. Each immersion exposure consisted of 400 µg steroid/l for 2 hr and feeding was at 3 mg steroid/kg diet for 60 days beginning with the onset of feeding. Immersions were applied one week prior (-1), during (0), or at one (+1) or two (+2) weeks after 50% hatch. Functional (Func.) males denotes percent of mature males with the ability to extrude milt from the gonopore. "--" denotes lost or discarded groups. Final yield for the MT, OHA, and control groups was 1.5, 1.6 and 74% respectively.

[1]Significantly different than pooled control all-female group: [a]$=P < 0.05$, [b]$=P < 0.01$, [c]$=P < 0.001$.

Figure 16. Gonads from XX males at two years of age that were sex-reversed by a single immersion in 17α-methyltestosterone. (A) Gonad at top is a near normal ovary with a small amount of testicular tissue at anterior end, two middle gonads are from an intersex fish with both testicular and ovarian tissue, bottom gonad is an apparently unaffected ovary. (B) Varying degrees of testicular formation, top and middle testes are small and less developed but still contain intact sperm ducts; bottom testis is well developed yet still 2/3 the size of a normal gonad.

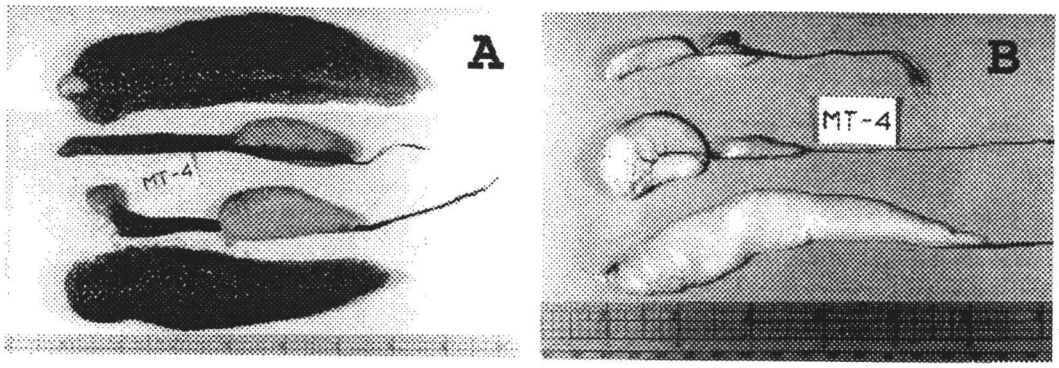

Figure 16.

of functional males (those with intact sperm ducts) while those both immersed in and fed MT did not. Groups 1, 5, 9, 10, 25, and 26 contained 100% functional males.

Progeny testing

Survival and female progeny produced from cryopreserved semen from the sex-reversed fish is given in Table II. The percentage survival, as compared to controls, ranged from 0-62%. Of the 23 XX males that sired offspring, 21 produced all-female progeny ($P < 0.001$ for all groups), one produced 71% females, and one produced 49%. Control animals produced 48% females.

DISCUSSION

A technique has been demonstrated for the production of stocks of all female rainbow trout which have been sex-reversed to create a source of "XX" sperm for further production of all female populations. The use of these stocks to indirectly produce all female populations could circumvent the problem of high mortality, which is normally seen when chromosomal (gynogenetic) manipulations are employed to directly produce all female populations. This technique, which requires only a brief immersion of embryos in MT or a combination of immersion plus feeding of the naturally occurring OHA, is simple, cost effective, and could be easily utilized in a hatchery setting. The use of

Table II. Percent survival (expressed as percent of control) and percent females of progeny produced from cryopreserved XX male semen. Each spawning trial consisted of 250 eggs fertilized with one ml of semen. Semen donor's group denotes treatment group from table 1. Differences in sex ratios between the control group and all spawnings except #2 and #20 are significant ($P < 0.001$).

Spawning	Semen donor's group	%Survival	%Females
1	3	22	100
2	24	21	71
3	3	48	100
4	3	61	100
5	2	38	100
6	25	60	100
7	25	60	100
8	6	55	100
9	13	24	100
10	2	37	100
11	2	0	--
12	7	3	100
13	3	62	100
14	25	36	100
15	3	62	100
16	6	23	100
17	3	43	100
18	3	39	100
19	6	12	100
20	6	47	49
21	6	0	--
22	24	15	100
23	3	29	100
24	22	48	100
25	6	0	--
26	13	32	100
27	Control	--	48

OHA may also alleviate the problems of marketing fish which have been exposed to synthetic steroids. Once stocks of rainbow trout bearing "XX" sperm have been produced, they can continue to generate all female populations for years. Sex-reversed gynogenetic fish also alleviate the necessity of breeding normal groups of sex-reversed fish to determine which animals are XX males.

Our finding of the labile period for androgen treatment of rainbow trout at 1 week post-hatch is in agreement with that of Piferrer and Donaldson (1989) who found similar results for coho salmon. Multiple immersions in MT decreased the numbers of males produced. It is possible that aromatization of MT occurred but this is unlikely given the extremely high doses of MT required for paradoxical feminization (Piferrer *et al.*, 1993).

Van den Hurk and van Oordt (1985) found that exposing rainbow trout to OHA resulted in transient masculinization when fish were either immersed in or fed the steroid. In the present study both immersion and feeding of OHA led to permanent masculinization. It is not known whether additive effects of these two treatments contributed to this condition or whether feeding alone could result in permanent masculinization. A permanent masculinizing effect of OHA when fed to coho salmon has been seen (Redding *et al.*, 1987).

Other studies attempting to produce sex-reversed males with intact sperm ducts by feeding fish lower doses of MT

have resulted in some success. Rainbow trout fed MT at 0.5 or 1 mg/kg diet for 60 days resulted in 82 and 66%, respectively, of the animals maturing functionally (Cousin-Gerber *et al.*, 1989; Tsumura *et al.*, 1991). We were able to achieve 100% functional males in several of the groups.

Two of the 26 sex-reversed males produced male offspring. A small percentage of male progeny produced by XX males has been documented before (Tsumura *et al.*, 1991). It is suspected that portions of the Y chromosome were not completely deactivated in UV irradiated sperm. The use of cryopreserved sperm from sex-reversed fish could alleviate the problem of male progeny by simply discarding sperm which does not produce 100% female populations.

Although the technique of immersing embryos in steroids during early development does not produce 100% monosex populations, our findings have indicated that a large proportion of the males that are produced using this method will be fully functional. This results in a large decrease in the labor required to maintain and spawn these fish. The use of the naturally occurring OHA will also produce functional males. Cryopreservation of semen from these animals could also lead to the production of all-female progeny for years.

ACKNOWLEDGEMENTS

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IV. ONTOGENY OF THE STRESS RESPONSE IN CHINOOK SALMON, *ONCORHYNCHUS TSHAWYTSCHA*

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ABSTRACT

Whole body concentrations of cortisol were determined via radioimmunoassay in chinook salmon, *Onchorynchus tshawytscha,* during early development in both stressed and non-stressed fish to determine when the corticosteroidogenic stress response first appeared. Progeny from both pooled and individual females were examined to determine if differences existed in offspring from different females. Progeny from pooled eggs showed relatively low levels of cortisol in eyed eggs, followed by an increase at hatching. Cortisol content of fry then decreased at 51 days postfertilization (dpf) then remained relatively constant for the duration of the study (86 dpf). Differences in cortisol between stress and control fish were first detectable 1 week after hatch (44 dpf) and persisted for the remainder of the study. The magnitude of the stress response, or relative amount of cortisol produced, generally increased from the time when it was first detected, but a decrease in the ability to elicit cortisol was seen at 65 dpf. Cortisol content of separate progeny from two individual females showed a similar pattern to that seen in pooled eggs with a decrease also observed at 51 dpf. A stress response was first detected 5 days post-hatch (42 dpf) and persisted for the remainder of the study. Differences were seen between progeny from the females when cortisol content was examined at 30 min after the stress event but not at 60 min. The magnitude of the stress

response generally increased during development, but a decrease was observed at 65 dpf. Our results indicate that chinook salmon are capable of producing cortisol following a stressful event approximately one week after the time of hatching. The decrease in endogenous cortisol content seen 2 weeks after hatching, and the decrease in the magnitude of the stress response seen 4 weeks after hatching may be comparable to developmental events documented in mammals where corticosteroid synthesis is inhibited to neutralize possible detrimental effects of these hormones during critical periods of development.

INTRODUCTION

Cortisol is the main steroid produced by most teleosts in response to stressful events. This corticosteroidogenic response is thought to be a mechanism by which the organism can both cope in the short term with the stressful event and as a means to maintain homeostasis after the stress. Longer periods of stress or cortisol production, however, can be deleterious (Barton and Iwama, 1991). Prolonged stress and elevated corticosteroid levels can have adverse effects on survival, growth, reproduction, immune function and general fitness (Specker and Schreck, 1980; Schreck, 1982; Maule *et al.*, 1989; Campbell *et al.*, 1994; McCormick *et al.*, 1998). A general knowledge of when fish first develop the ability to elicit a cortisol response and at which times during early development that they are particularly sensitive to stressful events could lead to more efficient handling methodologies for both aquaculture systems and management of wild populations.

Although several studies have examined endogenous cortisol content during early development in a variety of species (de Jesus *et al.*, 1991; de Jesus and Hirano, 1992; Hwang, P.P. *et al.*, 1992; Barry *et al.*, 1995a; Sampath-Kumar *et al.*, 1995; Yeoh *et al.*, 1996; Sampath-Kumar *et al.*, 1997), few have investigated when the ability to elicit a corticosteroidogenic response first occurs. Barry *et al.* (1995a) were able to detect a stress response in rainbow

trout, *Oncorhynchus mykiss*, at 42 days postfertilization (dpf) or two weeks after hatch. Pottinger and Mosuwe (1994) documented a corticosteroidogenic response in rainbow and brown trout, *Salmo trutta*, 5 weeks after hatching.

Further work by Barry *et al.* (1995b) showed through *in vitro* techniques that the hypothalamic-pituitary-interrenal (HPI) axis was intact prior to the time that fish could produce a cortisol stress response. They also found that the ability to produce cortisol following a stressful event decreased at four weeks after hatching and suggested that this was comparable to the stress hyporesponsive period (SHRP) seen in early mammalian development (Schapiro, 1962; review by Sapolsky and Meaney, 1986). During this period corticosteroid production is inhibited to neutralize the permanent deleterious effects that these hormones can have on development (Sapolsky and Meany, 1986; DeKloet *et al.*, 1988). They also suggested that it is during this time in trout when environmental perturbations can have permanent effects on the corticosteroidogenic stress response later in life.

The objectives of this study were to determine when in development chinook salmon, *O. tshawytscha*, first become capable of producing cortisol following a stressful event and to examine possible variation between fish by determining if differences existed in the timing or magnitude of the stress response between progeny from both pooled and individual females.

MATERIALS AND METHODS

Fish Rearing

Broodstock were obtained from the Oregon Department of Fish and Wildlife South Santiam hatchery, Sweet Home, and reared at Oregon State University's Fish Genetics and Performance Laboratory at Smith Farm, Corvallis, at a constant water temperature of 12 °C. We examined populations of chinook salmon from 2 brood years. For the first brood year (hereafter referred to as experiment 1) eggs from 6 females were pooled, fertilized by 3 males and then placed in Heath trays. Offspring were transferred to two-foot circular tanks with flow-through well water at 72 days postfertilization (dpf). Biomoist diet was fed from the onset of exogenous feeding 3 or 4 times daily to satiation.

For the second brood year (hereafter referred to as experiment 2) eggs from 2 females were kept separate and fertilized by pooled sperm from 2 males. Offspring from each female were kept separate and raised similarly to those from experiment 1.

Sampling

Offspring were stressed and sampled at 19 dpf (weak eyes), 26 and 33 dpf (strong eyes), 37 (50% hatch), 44 (100% hatch), and at 51, 58, 65, 70, 75, 79, and 86 dpf.

Developing embryos were stressed at each sampling date by placing eyed eggs into deoxygenated (1 ppm O_2) water which

had CO_2 bubbled through it for 30 min. This procedure caused no mortality nor did it reduce the temperature of the water. Control fish were left in Heath trays. Eggs (n= 15 for each group) were sampled 60 min after the end of the stress by placing them directly into liquid N_2.

At 50% hatch fish were stressed by holding them out of the water for 60 sec, placed back in heath trays and sampled 60 min later. At all other sampling times fish were held out of water for 60 sec, placed back in heath trays or tanks, then stressed again at three additional times by removing them from water for 30 sec at 15, 30, and 45 min after the original stressor. Fish were sampled 60 min after the 1st stressor (15 min after the last stressor).

To more precisely pinpoint the exact timing of appearance of the stress response, progeny from experiment 2 were sampled more frequently around the time when the ability to produce cortisol was determined from experiment 1. Offspring were sampled at 25 and 32 dpf (eyed eggs), 37 dpf (50% hatch), 39 dpf (100% hatch), and at 42, 44, 46, 49, 51, 58, 65, and 72 dpf.

Eyed eggs were stressed by placing them on paper towels for 20 min until the eggs started to "dimple". This procedure caused no mortality. Fish were stressed in the same manner as experiment 1 for the remaining sampling dates and an additional 15 fish were collected 30 min after the first stress at each date.

Extractions

Embryos to be analyzed for whole-body cortisol content were extracted with a method modified from Feist *et al.* (1990). Briefly, embryos were sonicated for 30 sec in 1.0 ml of 0.5N NaOH and 50µl of acetic acid was added to lower the pH to approximately 4.9 to enhance efficiency of cortisol extraction. Homogenates were extracted twice with 8 vol of diethyl ether. The aqueous phase was removed by snap freezing in liquid nitrogen. Combined extracts were dried in a Speed Vac centrifuge. Extraction efficiencies were determined at each sampling date (n=6) by adding tritiated cortisol to tubes containing whole fish that were then sonicated and extracted as described above. The average extraction efficiencies ranged from 66.8 to 77.6%. All assays were corrected for recovery. Extracts to be analyzed for cortisol content were resuspended in 1.0 ml of phosphate-buffered saline with gelatin (PBSG)and 0.1 ml aliquots were removed for RIA.

Assays

Whole body cortisol content was measured following the method of Redding *et al.* (1984) with slight modifications. All standards and unknowns were reconstituted in PBSG and a heat denaturing step was omitted. Concentrations of both antibody and tracer were halved to increase sensitivity of the assay. The lower limit of detection was 5 pg/tube. The inter- and intra-assay coefficients of variation for all

assays was less than 5 and 10% respectively. Steroid levels determined by RIA were validated by verifying that serial dilutions were parallel to standard curves.

Statistics

Data for cortisol content between control and stressed groups were analyzed by analysis of variance. The level of significance for all tests was $P < 0.05$ for Fisher's protected least significant difference.

RESULTS

Experiment 1.

Cortisol content of fish from 19 to 86 dpf is shown in Fig. 17. Steroid levels were relatively low (1-2 ng/gm fish) in eyed eggs but increased significantly at the time of hatch (37 dpf). These levels decreased 2 weeks after hatch, then rose for 2 weeks and then declined for 10 days where they remained relatively constant for the duration of the study. Significant differences in cortisol content between stressed and control groups were first observed 1 week after hatch and continued for the duration of the study. The "magnitude" of the stress response (ratio of cortisol levels in stressed vs. controls-Fig. 18) generally increased from the time when fish could elicit a response to the end of the study. There was a decrease, however, in this ability at 65 dpf.

Figure 17. Cortisol concentrations (pg/gm) in stressed and non-stressed (control) chinook salmon from 19 to 86 days postfertilization from experiment 1. Each value represents the mean $\pm$ SE for a sample size of 15. "*" denotes statistically significant differences from controls for an ANOVA test with $P < 0.05$.

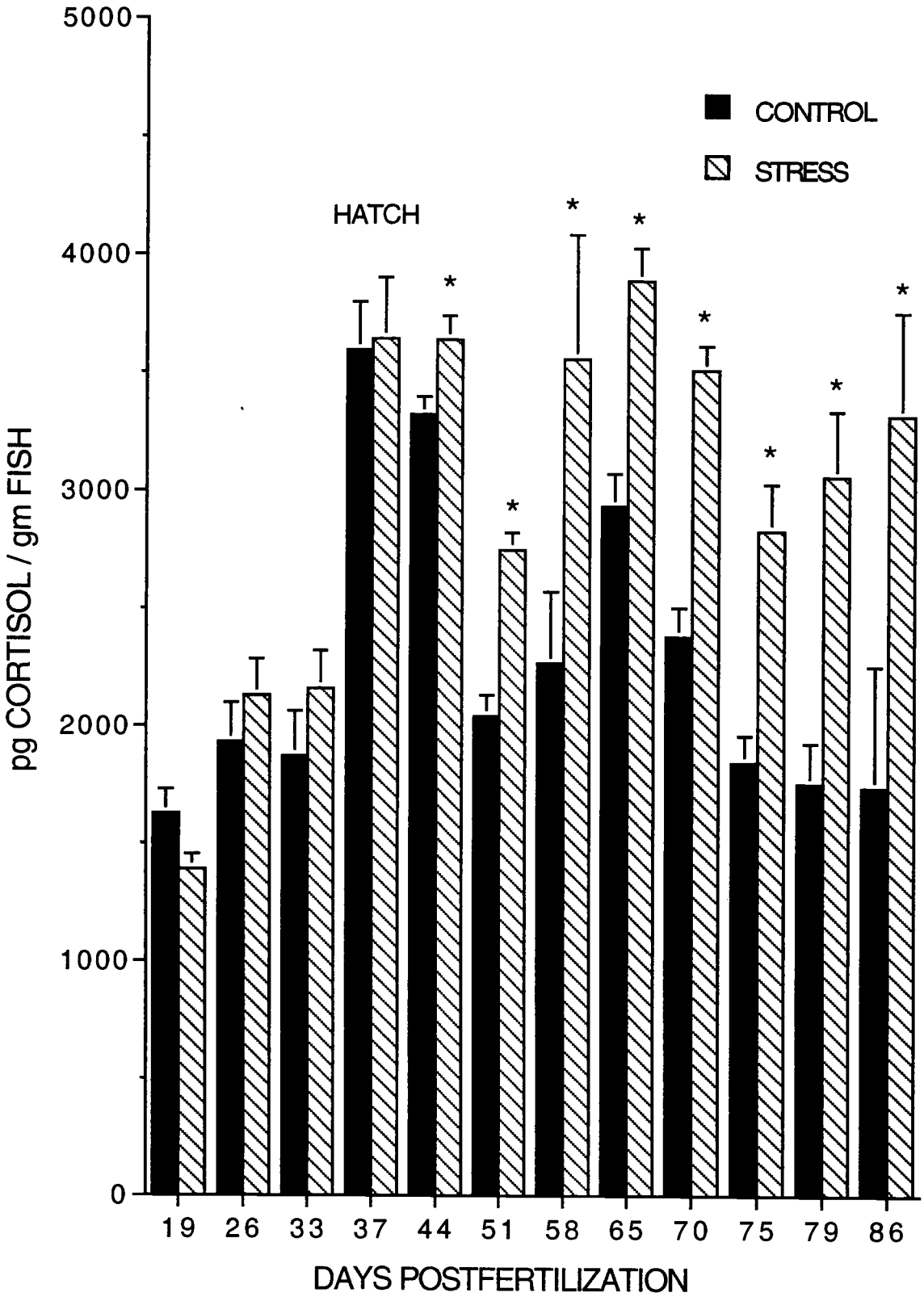
5000
4000
3000
2000
1000
0
pg CORTISOL / gm FISH
CONTROL
STRESS
HATCH
19
26
33
37
44
51
58
65
70
75
79
86
DAYS POSTFERTILIZATION

Figure 17.

Figure 18. Magnitude of the stress response in chinook salmon from 19 to 86 days postfertilization from experiment 1 expressed as a ratio of cortisol concentrations (pg/gm) in stressed vs. control fish.

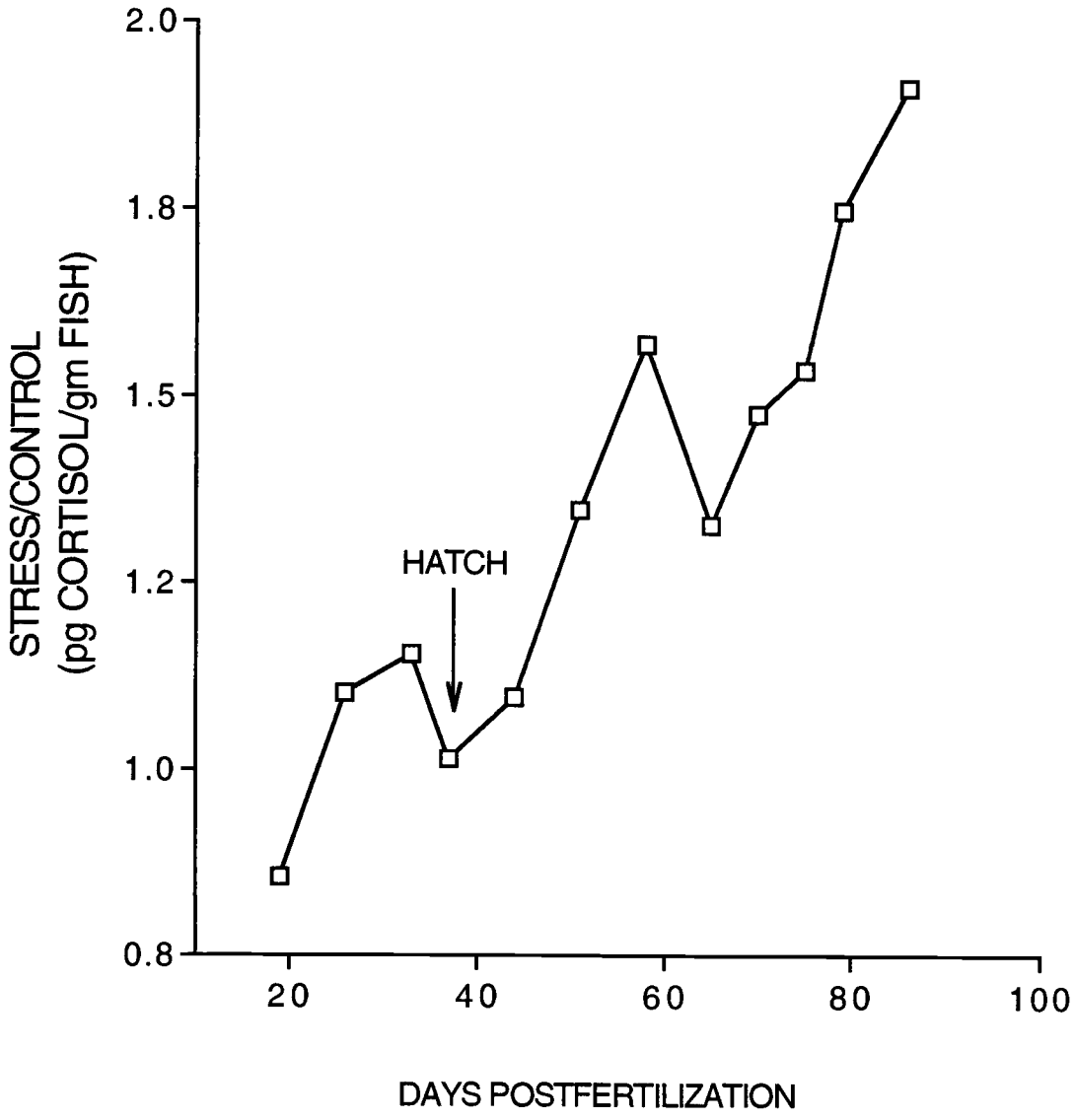
2.0
1.8
1.5
1.2
1.0
0.8
STRESS/CONTROL
(pg CORTISOL/gm FISH)
HATCH
20
40
60
80
100
DAYS POSTFERTILIZATION

Figure 18.

Experiment 2, Female 1

Cortisol content of progeny from female 1 from 25 to 72 dpf is shown in Fig 19. Cortisol levels were relatively low (approximately 2 ng/gm fish) in eyed eggs and then increased slightly at hatch (37 dpf) and remained relatively constant. These levels declined dramatically 2 weeks after hatch then rose one week later and remained at relatively uniform levels. Significant differences in cortisol between stressed and control fish were first seen 5 days after hatch, when examined 60 min after the handling stresses, and levels were significantly different for the remainder of the study. When examined 30 min after the handling stresses, significant differences in cortisol were first seen 9 days after hatching but were not always seen throughout the remainder of the study. There was an insufficient number of fish to obtain a sample at 30 min post-stress for the last sampling date (72 dpf). The magnitude of the stress response over time is shown in Fig. 20. The pattern seen was remarkably similar to that in experiment 1 with increasing cortisol produced as development proceeded (at both 30 and 60 min post-stress) and a marked decline was seen at 65 dpf.

Experiment 2, Female 2

Cortisol content of progeny from female 2 from 25 to 72 dpf is shown in Fig. 21. Cortisol levels were very similar to those seen in Female 1 with the exception that no

Figure 19. Cortisol concentrations (pg/gm) in stressed and non-stressed (control) chinook salmon progeny from female 1, experiment 2 from 25 to 72 days postfertilization . Each value represents the mean $\pm$ SE for a sample size of 15. a and b denote statistically different from controls and 30 min post- stress fish, respectively, for an ANOVA test with $P < 0.05$.

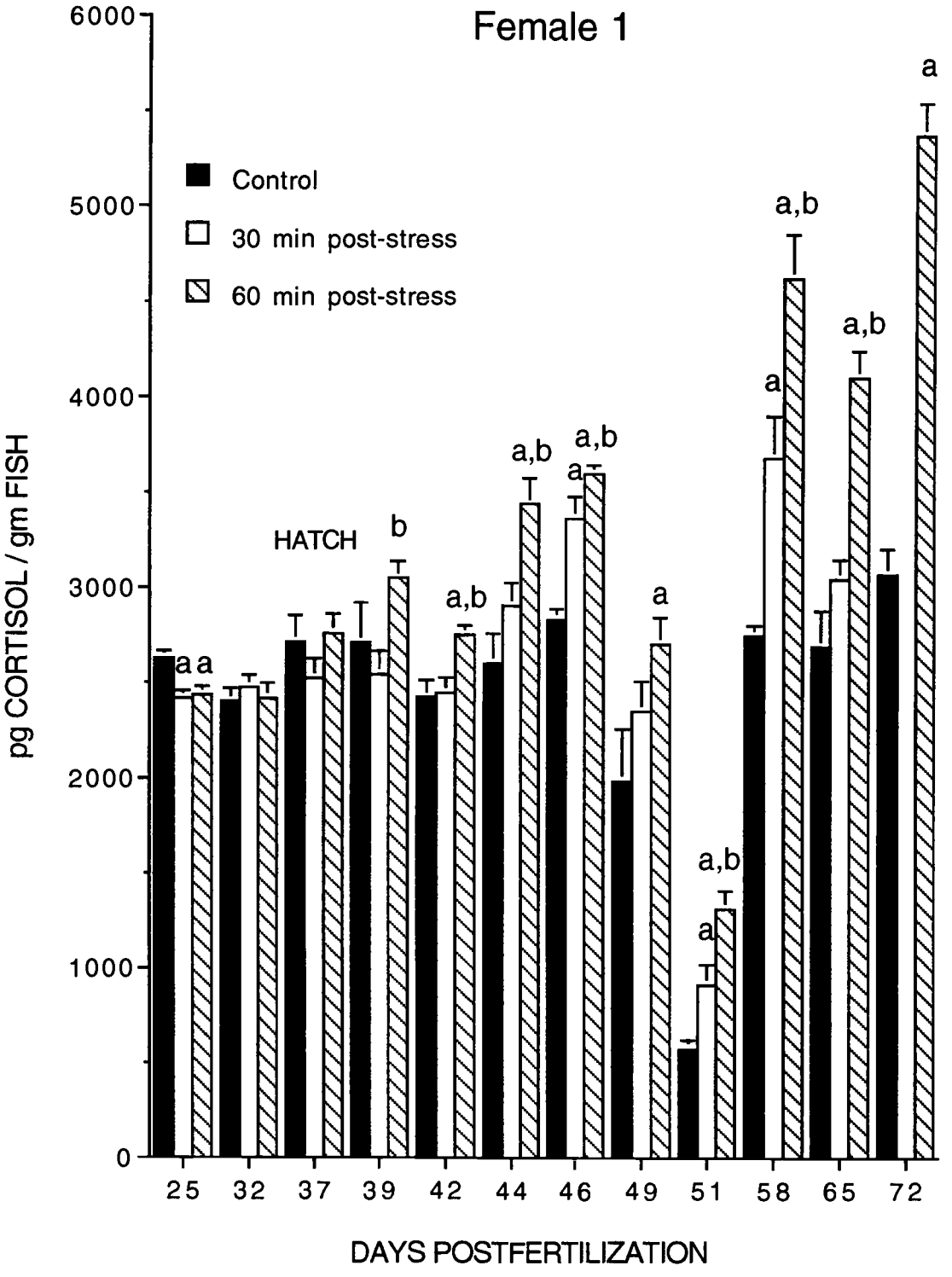
Female 1
Control
30 min post-stress
60 min post-stress
pg CORTISOL / gm FISH
6000
5000
4000
3000
2000
1000
0
HATCH
a a
b
a,b
a,b
a,b
a
a
a,b
a
a,b
a,b
a
25
32
37
39
42
44
46
49
51
58
65
72
DAYS POSTFERTILIZATION

Figure 19.

Figure 20. Magnitude of the stress response in chinook salmon from females 1 and 2, experiment 2, at both 30 and 60 min post-stress from 25 to 72 days postfertilization. The magnitude is expressed as a ratio of cortisol concentrations (pg/gm) in stressed vs. control fish.

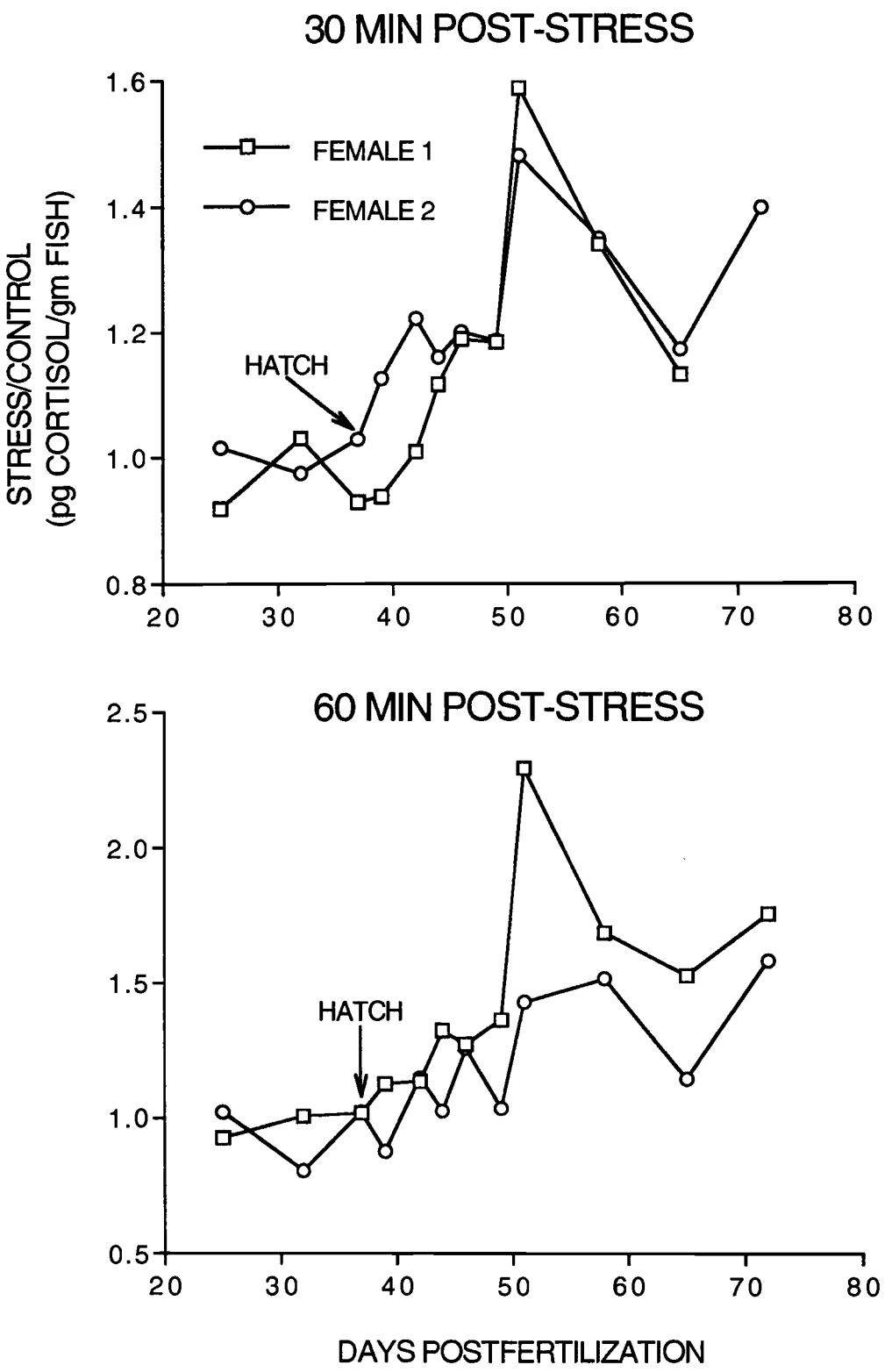
30 MIN POST-STRESS
STRESS/CONTROL
(pg CORTISOL/gm FISH)
FEMALE 1
FEMALE 2
HATCH
1.6
1.4
1.2
1.0
0.8
20
30
40
50
60
70
80
60 MIN POST-STRESS
2.5
2.0
1.5
1.0
0.5
HATCH
DAYS POSTFERTILIZATION

Figure 20.

Figure 21. Cortisol concentrations (pg/gm) in stressed and non-stressed (control) chinook salmon progeny from female 2, experiment 2 from 25 to 72 days postfertilization. Each value represents the mean $\pm$ SE for a sample size of 15. a and b denote statistically different from controls and 30 min post- stress fish, respectively, for an ANOVA test with $P < 0.05$.

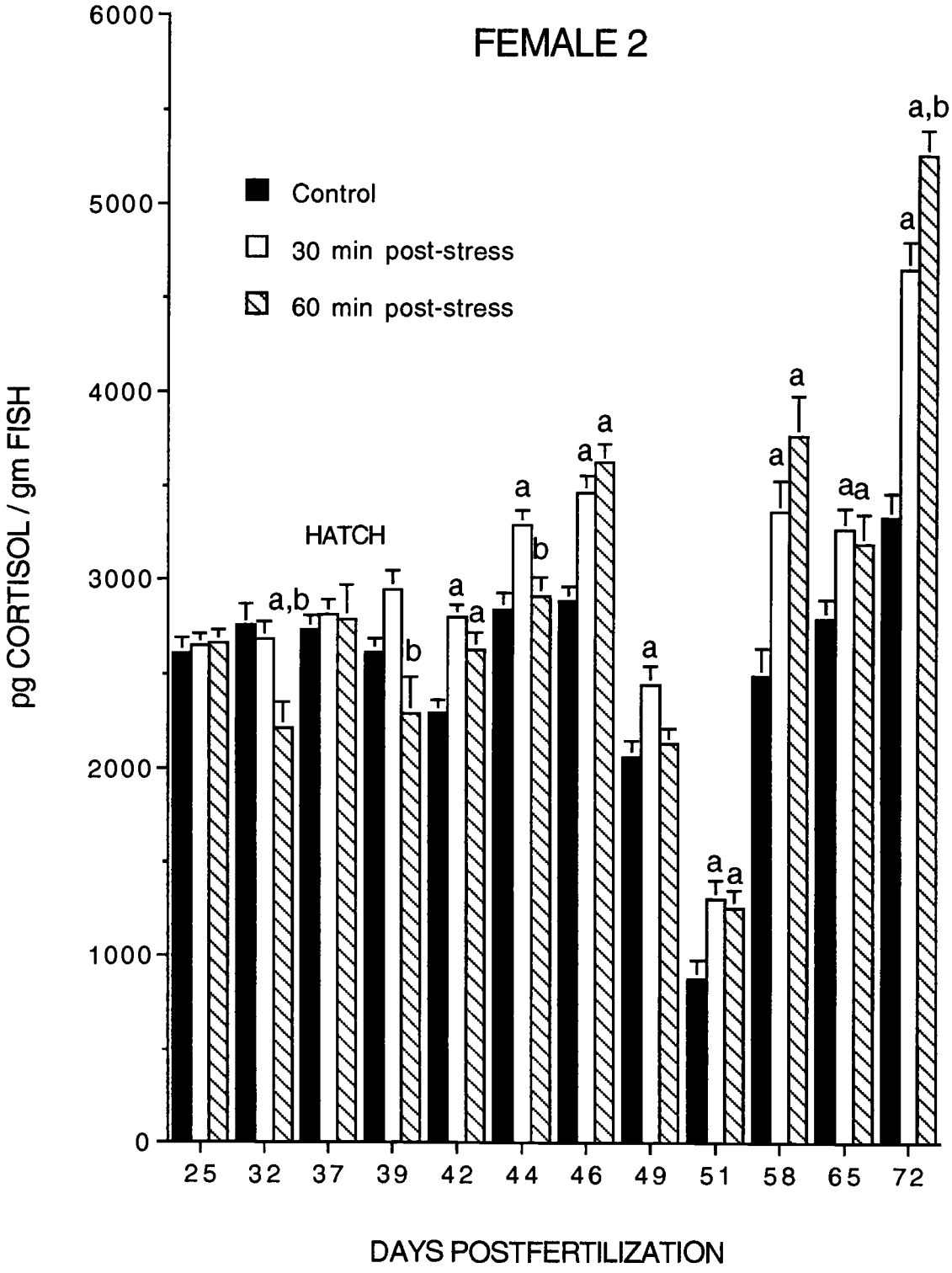
FEMALE 2
Control
30 min post-stress
60 min post-stress
HATCH
pg CORTISOL / gm FISH
6000
5000
4000
3000
2000
1000
0
25
32
37
39
42
44
46
49
51
58
65
72
DAYS POSTFERTILIZATION

Figure 21.

increase was seen at hatch. A decline in cortisol content was also seen at 51 dpf followed by increases for the last three weeks of the study. Significant differences in cortisol between stressed and control fish were first seen 5 days after hatch, the same as in Female 1. Unlike Female 1 cortisol was consistently elevated at both 30 and 60 min post-stress (when compared to controls) but only one sampling date (72 dpf) showed an increase from 30 to 60 min post-stress. The magnitude of the stress response (Fig. 20) was very similar to that seen in female 1 with the amount of cortisol elicited generally increasing throughout development and a decrease was seen at 65 dpf.

DISCUSSION

Cortisol was found at all stages of development examined in this study and it appears that chinook salmon are capable of producing this steroid around the time of hatching with a corticosteroidogenic stress response developing about one week later. Endogenous cortisol has been measured in a variety of teleosts during earlier development which have shown a general pattern of relatively high levels after fertilization followed by a decline until the time of hatching. It is believed that the cortisol is of maternal origin and that embryos are metabolizing the steroid during early development (de Jesus *et al.*, 1991; Hwang *et al.*, 1992; Sampath-Kumar *et al.*, 1997).

Our finding of relatively low cortisol concentrations in eyed eggs followed by an increase around the time of hatching has also been documented in the Japanese flounder, *Paralichthys olivaceus*, (de Jesus *et al.*, 1991), tilapia, *Oreochromis mossambicus*, and milkfish, *Chanos chanos*, (Hwang *et al.*, 1992), chum salmon, *O. keta*, (de Jesus and Hirano, 1992), rainbow trout, *O. mykiss*, (Barry *et al.*, 1995), and Asian seabass, *Lates calcarifer*, (Sampath-Kumar *et al.*, 1995).

Studies involving radioactive precursors have shown that Asian seabass hatchlings could convert them into cortisol (Sampath-Kumar *et al.*, 1997). Using immunocytochemistry techniques these researchers demonstrated both the presence of steroid synthesizing enzymes in interrenal tissue and ACTH in the pituitary. Barry *et al.* (1995) used *in vitro* techniques to demonstrate that alevin rainbow trout interrenal tissue could respond to ACTH by producing cortisol. These studies indicate that the pituitary-interrenal axis is intact at or before hatching and that increases in steroids around the time of hatching are a result of de novo synthesis.

Our findings indicate that the HPI axis is intact as early as 5 to 7 days after hatch. It is possible that the fish in our study developed a cortisol stress response prior to the time that we were able to detect it but the comparatively high residual levels of maternal cortisol obscured it. Barry *et al.* (1995a,b) documented a stress

response in rainbow trout 2 weeks after hatch while Pottinger and Mosuwe (1994) showed a response in rainbow and brown trout 5 weeks after hatching. The differences in timing of appearance of the stress response could be due to species differences, rearing conditions, assay sensitivities or types of stressors applied.

Barry *et al.* (1995a,b) reared their fish at 10 °C so the stress response appeared at 420 degree days of development. In our study it appeared around 500 degree days. These researchers employed a handling stress followed by a 30 sec cold shock. This decrease in temperature may have slowed the rate at which enzyme systems were synthesizing steroids and may have obscured subtle changes in cortisol content even earlier in development. Pottinger and Mosuwe (1994) reared their fish at a range of 5 to 16 °C. Relatively cooler temperatures during earlier development may have delayed the onset of the stress response. These researchers employed a netting and confinement stress which may not have been as severe as the handling stress in our study.

Although similar patterns in whole body cortisol content were seen between brood years and individual females, some differences were detected. A relatively large increase in cortisol at hatch was seen in progeny from pooled eggs, while progeny from female 1 showed only a slight increase and progeny from female 2 showed no increase. Progeny from female 2 showed consistently elevated

cortisol at 30 min post-stress while those from female 1 did not. Progeny from female 1 were able to produce additional cortisol between 30 and 60 min post-stress while those from female 2 did not. These subtle differences should not be surprising. It is well accepted that a large amount of variation exists with regard to the magnitude of the stress response among both individuals and populations (e.g. Pottinger and Moran, 1993, Pottinger and Carrick 1999).

It also seems that some of this variation could be genetically linked. Pottinger *et al.* (1994) found that progeny from parents with a relatively high cortisol response following stress produced more of the steroid than progeny from parents with a relatively low response. The degree to which rainbow trout produced cortisol in response to stress could also be modified by selective breeding (Pottinger and Carrick, 1999).

We found a consistent pattern of endogenous cortisol concentrations, appearance of the stress response and changes in the relative magnitude of this response among two brood years and for both pooled eggs and progeny from two individual females. The decreases in both endogenous cortisol at 51 dpf and of the magnitude of the stress response 2 weeks later may be indicative of control mechanisms by fish to down regulate cortisol titres during periods of development when larvae are particularly susceptible to detrimental effects of corticosteroids. Barry *et al.* (1995a,b) found very similar results with rainbow

trout and suggested that this period in development was comparable to the SHRP seen in mammals.

This period is thought to be an adaptive mechanism to compensate for the deleterious effects that corticosteroids can have on growth and development of the central nervous system during critical periods of neuronal integration (Sapolsky and Meaney, 1986). It is also during this time when severe or multiple stressful events can have long-lasting or permanent effects on morphology, physiology and behavior and can alter the way organisms respond to stress as adults (de Kloet *et al.*, 1988).

It is for these reasons that care must be taken when handling fish in aquaculture or wild settings during these critical periods of development. Realizing that salmonids become capable of a corticosteroidogenic response shortly after hatching and, although they can compensate for deleterious effects of cortisol to some degree, may become very susceptible to prolonged or severe stressful events four weeks later should lead to more efficient and less stressful handling methodologies. It is interesting to note that chinook salmon in hatcheries typically reach the swim-up stage around four weeks post-hatching and it is at this time that they are transferred from incubation trays to tanks and the onset of feeding begins. In the wild, depending on water temperature, fry also begin to emerge from gravel around this time. A SHRP during this time may be an adaptation to down regulate, but still leave in place,

the stress response when organisms are particularly challenged by new environmental settings during critical periods of neuronal development.

ACKNOWLEDGEMENTS

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V: CONCLUSION

Our objectives in these studies were to elucidate possible endocrine control mechanisms of sexual differentiation in salmonids, to determine more effective methodologies for production of XX males and to examine at what stage of development salmonids become capable of a corticosteroidogenic stress response. A finding common to the three studies was that both endogenous steroid levels and the effects of exogenous steroids became labile during two periods of development, namely hatching and onset of feeding. Questions immediately arise as to the function of steroids during these periods and their mechanism of action.

If Yamamoto's (1969) hypothesis that steroids are the inducers of sex differentiation in fishes is correct, one would expect to see a scenario similar to the findings of our first study (Chapter II). Our results showed that sex steroids increased around the time of hatching and became sexually dimorphic during the period of gonadal differentiation. There are multitudes of chromosomal systems in fishes, and no sex-specific gene product for either testis or ovarian differentiation has been found. These observations coupled with numerous studies documenting de novo synthesis of steroids at or before the period of gonadal differentiation points to steroids as being the main contributors to differentiation.

Steroids during early development may preprogram the animal for both reproductive and nonreproductive requirements seen later in life. Steroids could exert their effects in a variety of ways: they may induce or inhibit the appropriate steroid receptors and synthesizing enzymes, depending on the sex of the animal, to bring about the proper tissue organization for masculinization or feminization. In mammals, gonadal steroids during early development can induce permanent changes in the CNS (Maclosky and Naftolin, 1981). It is possible that sex steroids in fish influence development of the BPGA by bringing about changes that will lead to the complex feedback systems seen later in life. Sex differences, with regard to both localization and content of GnRH and the gonadotropins, have been documented in adult fish (Gentile *et al.*, 1986; Elofsson *et al.*, 1999; Gen *et al.*, 2000). Steroid effects on protein hormones in the brain and pituitary during the period of sexual differentiation could be reflected in adult animals during the time of maturation.

Steroids during early development may also effect processes other than sex differentiation, including behavior. Sex steroids may in part control permanent changes in neuronal networks during imprinting of young salmon to their natal streams. Changes induced in the BPGA by steroids during this period may have long lasting effects on future behavior. Homing migration in salmonids is thought to be influenced by the BPGA (Sato *et al.*, 1997; Ueda *et al.*,

1999), and the effect of steroids during imprinting may be responsible for behaviors seen much later in life.

Although it appears that sex steroids are intimately involved in sex differentiation, it is obvious that at some point there must be genetic differences between males and females that yield protein products that control which steroids are synthesized or direct the primordial gonad to differentiate. This could be due to sex differences in either single or multi-loci expression of steroidogenic enzymes or different steroid receptors or a combination of both (see Nagahama, 1999). Of course the possibility of other novel mechanisms of differentiation exist, and given the wide variety of reproductive strategies that fish utilize, it would not be surprising to find many types or variations of differentiation among species.

In our second study (Chapter III), the sex reversing effects of steroids became labile during the periods of hatching and onset of feeding. It was during these same developmental periods in the first study that endogenous steroids became either dynamic or sexually dimorphic. It is possible that both exogenously administered and endogenous steroids are exerting their effects on sex differentiation during these times. It also appears that a relatively high or prolonged dose of exogenous steroid is required to override the actions of naturally occurring hormones.

The most likely mechanistic explanation for the sex reversing effects of steroids would be receptor mediated.

Fitpatrick *et al.* (1994) have documented androgen receptors in the ovaries of juvenile coho salmon, and Gale (1996) found them in both ovaries and testis of tilapia. The gonads would be the most likely site for the effects of steroids, but many other tissues contain androgen and estrogen receptors and the possibility of extra-gonadal influence cannot be discounted.

Following exposure of fish to steroids to induce sex reversal, one would expect protein products specific to steroidogenesis or gonadal development to be induced or repressed, depending on the genetic sex of the fish and the type of compound applied. Fitzpatrick (1990) found a female specific gonadal protein in both young coho salmon and rainbow trout. Dietary treatment with an androgen to cause sex reversal of trout resulted in the reduction or absence of this protein in an all-female population. Recently, Contreras-Sanchez *et al.* (2000) employed subtraction hybridization techniques to examine mRNA and protein products of tilapia exposed to a synthetic androgen. The steroid induced the expression of a number of unique genes, including 11 that were related to reproductive tissues. Several of the identified protein sequences were similar to those found in mammals that have been linked to gonadal development, testicular protein synthesis, steroid mobilization and cell growth. Additional studies of this nature need to be conducted during both induced sex reversal and in animals undergoing the normal process of

differentiation to precisely define the sequence of events leading to a functional testis or ovary.

It is hoped that techniques to produce functional sex-reversed fish, such as the ones developed here, could circumvent the problems of high mortality associated with chromosomal manipulations, high cost and time-consuming nature of the techniques that are currently being used and the deleterious effects of surgery on broodstock. Shorter exposure times and the use of naturally occurring steroids may decrease possible risks to human workers in aquaculture facilities and the environment and alleviate the problems of marketing fish which have been exposed to synthetic hormones.

Our third study (Chapter IV) showed that chinook salmon became capable of producing cortisol around the time of hatch and that they could elicit the steroid following a stressful event approximately one week later. Decreases in both endogenous cortisol content and the magnitude of the stress response, seen at 2 and 4 weeks after hatching respectively, may be indicative of a developmental period in fish that is comparable to the SHRP in mammals (Barry *et al.*, 1995a,b). If this is indeed a compensatory mechanism to inhibit the deleterious effects of corticosteroids on neuronal integration or other developmental events (Sapolsky and Meaney, 1986; de Kloet *et al.*, 1988), the question arises as to what the events are.

One candidate for an early developmental event that could be negatively effected by cortisol is proliferation of the immune system. Cortisol can have deleterious effects on immune function (Maule *et al.*, 1989), and down-regulation of both endogenous cortisol and the ability to produce it during stress may be a mechanism to allow for proper development of the immune system. Studies examining ontogeny of lymphoid organs and appearance of immunoglobulins in salmonids have found that the thymus and kidney generally become lymphoid around the time of hatching, while the spleen develops later, and the ability to produce immunoglobulins does not occur until the onset of feeding (Ellis, 1977; Grace and Manning, 1980; Razquin *et al.*, 1990).

It was during the two developmental periods of hatching and onset of feeding that both whole body cortisol content and production of the steroid following a stress became labile. It is generally thought that relatively high levels of cortisol are anti-developmental in nature but that low levels of the hormone are needed to provide a permissive role in growth and development (Schreck, 1992). It is possible that the relatively low levels of cortisol during the period of hatching are playing a permissive role to enhance growth. Inhibition of the stress response seen later may be an adaptive mechanism to circumvent the deleterious effects of high levels of cortisol during development of the immune response. Hence, lowering cortisol production at

critical times could be "permissive" to development. There may also be interactive effects between the sex steroids and cortisol during developmental periods of tissue formation or reorganization to down regulate possible autoimmune responses.

In recent years, concern has risen dramatically over the possible effects of endocrine disrupting chemicals (EDCs) or "hormone mimics" on wild populations of fish. Studies such as ours may prove invaluable for delineating the early stages of development in which fish are particularly sensitive to the effects of these compounds. Screening methodologies could then be developed to test the effects of a variety of toxicants. Exposure of animals to EDCs during early development may also provide additional insight to possible mechanisms of sex differentiation in fish. Given their varied reproductive strategies, including the entire range of hemaphroditism and natural sex reversal, fish can provide us with a seemingly endless number of model systems to manipulate.

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